Max-Planck-Institute of Molecular Plant Physiology Golm

Analysis of transcription factors under sulphur deficiency stress

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This Ph.D. thesis is the result of my own work and was done between October 2002 and December 2005 in the department of Prof. Dr. Lothar Willmitzer at the Max-Planck-Institute of Molecular Plant Physiology in Golm, Germany. It has not been submitted for any degree or Ph.D. at any other university.

Die Dissertation ist das Ergebnis praktischer Arbeit, welche von Oktober 2002 bis Dezember 2005 durchgeführt wurde im Department von Prof. Dr. Lothar Willmitzer im Max-Planck-Insitut für Molekulare Pflanzenphysiologie, Golm, Deutschland. Ich versichere, daß ich die vorliegende Arbeit selbständig verfaßt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Diese Dissertation wurde an keiner anderen Hochschule zu Prüfung eingereicht.

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CONTENT

1 INTRODUCTION

1.1 Sulphate metabolism in higher plants

1.1.1 The biochemical role of sulphur

Sulphur, a macronutrient essential for plant growth, is among the most versatile elements in living organisms. The versatility of sulphur derives in part from the property that it shares with nitrogen: multiple stable oxidation states (Hell, 1997). Sulphur is present at approximately 0,1% of the dry matter of plants.

Elemental sulphur is essential for photosynthetic and respiratory electron transport through Fe-S clusters. Reduced sulphur is found in wide range of biological compounds, such as two sulphur-containing amino acids, cysteine (Cys) and methionine (Met), oligopeptide glutathione (GSH) and phytochelatins, polymerised forms of GSH. Several coenzymes and vitamins, including coenzyme A, *S*-adenosyl-L-methionine (SAM), thiamine, biotin and *S*-methylmethionine (SMM) contain functionally important sulphur moieties. SAM itself has key functions as a primary methyl-group donor used in RNA and DNA modifications, and as a precursor for metabolites such as ethylene, polyamines, vitamin B1, 3-dimethylsulphoniopropionate (an osmoprotectant), and as a source of atmospheric sulphur: dimethylsulphide (Amir et al., 2002, Hesse et al., 2004b). SMM is used as a major transport molecule for reduced sulphur in some plants, connecting sink and source organs (Bourgis et al., 1999). Chloroplast membranes contain a sulpholipid, sulphuquinovosyldiacylglycerol. Some signalling molecules contain sulphur as a key component, including sulphated lipooligosaccharides that function as rhizobial Nod factors, and turgorin, which is responsible for thigmotactic movement in leaves of *Mimosa pudica*. Secondary metabolites that contain sulphur range from produced by *Brassica* defense compounds, glucosinolates, large and diverse group of flavonoids to antiseptic allin in garlic and anticarcinogenic sulphophane in broccoli. Phytochelatins are involved in detoxification of heavy metals by serving as chelating ligands through thiol groups (Rauser, 1995). Glutathione xenobiotic conjugates are transported into the vacoule for deposition (Tommasini et al., 1998). Additionally, GSH acts as a stress signal and can regulate plant development (May et al., 1998).

As a part of the Cys molecule, the sulphur group, named the thiol-group, is strongly nucleophilic (electron donating), making it ideally suited for biological redox processes. When oxidized, two Cys molecules can form a covalent linkage called a disulphide bond, which is readily broken by reduction to form two thiol groups. Disulphide/dithiol interchange is so versatile that nearly all aerobic forms of life, including plants, use this reaction as the dominant form of redox control. Redox control regulates enzymes and protects against oxidative damage (Leustek and Saito, 1999).

Free Cys is not used for redox control as it is much too readily oxidized to cystine. A variety of more stable thiol compounds are involved in redox regulation. The most abundant one is glutathione, an enzymatically synthesized tripeptide in which Cys is linked via peptide bonds to the γ-carboxyl group of Glu and the α-amino group of Gly. The balance between reduced and oxidized form of glutathione (GSH/GSSG) is maintained by the enzyme glutathione reductase, using NADPH as an electron source. This allows to keep the plant cytoplasm, chloroplast stroma, and mitochondrial matrix highly buffered in the reducing state. Many intracellular enzymes require reducing conditions for their activity, because Cys residues in proteins can also form disulphide bonds, resulting in a disruption of structure and a loss of activity. There are special cases in which specific disulphide bonds are required for formation of tertiary and quaternary structure in a protein, but this is less common, especially for soluble intracellular proteins (Leustek and Saito, 1999).

Plant sulphur assimilation plays a key role in the sulphur cycle in nature. Animals, including humans, do not posses the mechanism for sulphur assimilation being unable to reduce sulphur and therefore require dietary sources of methionine as an essential amino acid (Hesse and Hoefgen, 2003; Hesse et al., 2004b).

1.1.2 Sulphate transport and assimilation

Sulphur is taken up by plants in its inorganic sulphate form. Transport accross the plasma membrane is driven by electrochemical gradient generated by an ATP-dependent proton pump (Saito, 2000). The sulphate transporter is a symporter that transports one sulphate molecule in exchange for three protons into the cell. Plant sulphate transporter genes are predicted to have 12 membrane-spanning domains creating a channel across the plasma membrane (Smith et al., 2000). Sulphate transporters localized in the root plasma membrane mediate uptake from external environments (the soil or apoplasts) into the symplastic system. Plasma-membrane transport systems for xylem loading in roots and unloading in leaves are also necessary. Once inside plant cells, sulphate is transported by organelle-membrane transport systems within the chloroplast envelopes and tonoplast membranes (Saito, 2000).

To facilitate the complex movements of sulphate around the plant, the sulphate transporters themselves are encoded by a gene family consisting of 14 members in *Arabidopsis*, probably with little redundancy (Hawkesford, 2003). Based on sequence similarities, tissue and cellular localisation and kinetic properties, various sulphate transporters were classified into 5 groups named *Sultr1 – 5* (Buchner et al., 2004). High-affinity forms of the group 1 (*Sultr1;1*, *Sultr1;2*) are predominantly but not exclusively expressed in roots, whereas the lower-affinity forms (*Sultr2;1*, *Sultr2;2*) are expressed in vascular tissues, principally in leaves but also in roots (Smith et al., 1995; 1997). The expression pattern of the high-affinity type suggests that it mediates uptake of SO_4^{2-} into the plant and is a way to adjust to variation in the external sulphur supply. By contrast, low-affinity transporters could function in SO_4^{2-} uptake, both from soil and from the apoplast solution that bathes internal cells (Leustek and Saito, 1999).

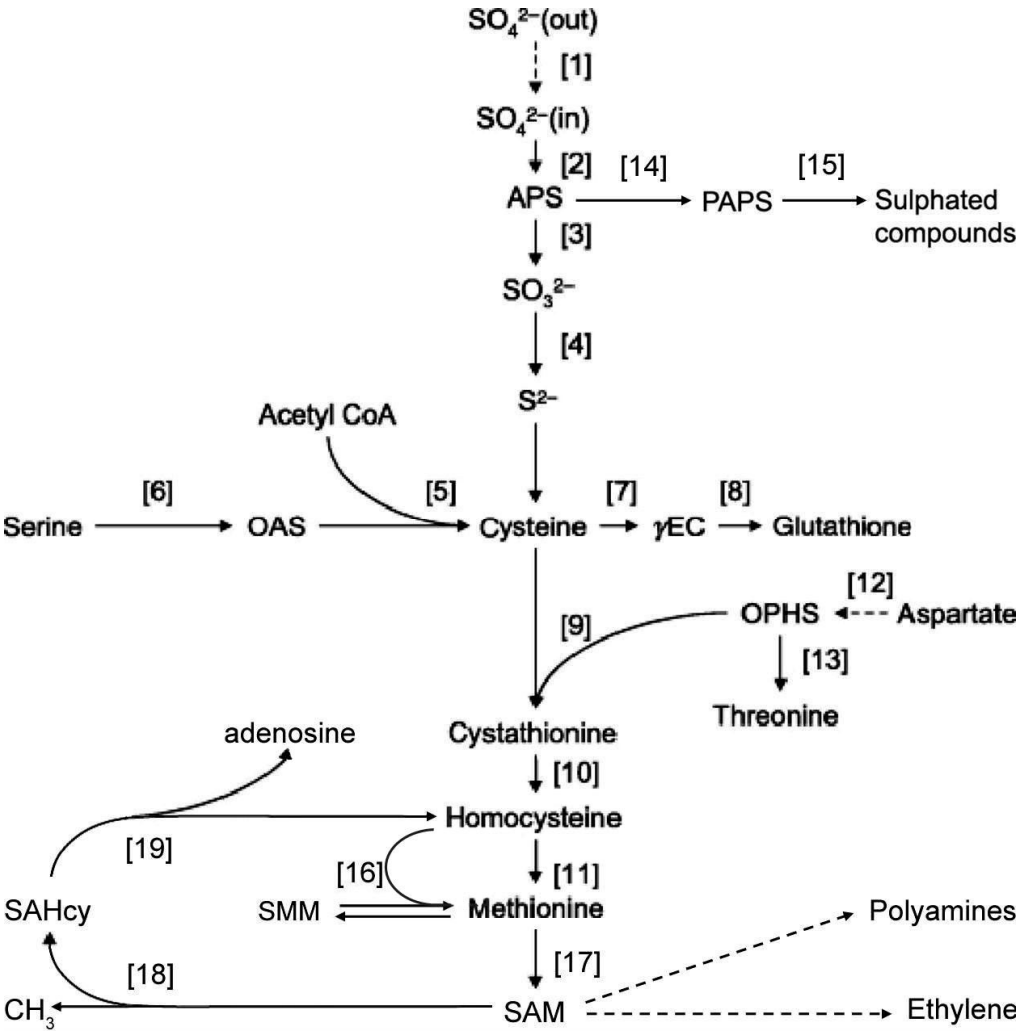

Fig 1-1 Biosynthetic pathways of the sulphur-containing amino acids, cysteine and methionine, and the sulphation pathway – two distinct ways existing in plants for sulphate incorporation into organic compounds (redrawn from Bick and Leustek, 1998; Ravanel et al., 1998; and Hawkesford and De Kok, 2006).
[1] sulphate transporters; [2] ATP sulphurylase; [3] adenosine phosphosulphate (APS) reductase; [4] sulphite reductase; [5] *O*-acetylserine (OAS) (thiol)lyase; [6] serine acetyltransferase; [7] γ-glutamylcysteine synthetase; [8] glutathione synthetase; [9] cystathionine γ-synthase; [10] cystathionine β-lyase; [11] methionine synthase; [12] aspartate kinase, aspartate semialdehyde dehydrogenase, homoserine kinase; [13] threonine synthase; [14] APS kinase; [15] sulphotransferases; [16] homocysteine S-methyltransferase; [17] S-adenosylmethionine synthetase; [18] SAM-dependent methylase; [19] S-adenosylhomocysteine hydrolase. APS, adenosine-5'-phosphosulphate; PAPS, 3-phosphoadenosine-5'-phosphosulphate; CoA, coenzyme A; γ-EC, γ-glutamylcysteine; OPHS, *O*-phosphohomoserine; SMM, S-methylmethionine; SAM, S-adenosylmethionine; SAHcy, S-adenosylhomocysteine.

Group 3 of sulphate transporters do not appear to be regulated by sulphur nutritional status and their expression seems to be restricted to leaves (Takahashi et al., 2000). Groups 4 and 5 sulphate transporters have been localized to the tonoplast membrane (Kataoka et al., 2004b). The group 4 transporters have been implicated in efflux of sulphate from vacuole and are up-regulated by sulphur stress, thus favouring the unloading of sulphate from the vacuole. The role of the group 5 transporters has yet to be established (Hawkesford and De Kok, 2006).

A system that transports sulphate into plastids is necessary because plastids are the exclusive sites for the reduction of sulphate into sulphide. The protein sequences of all group 4 transporters contain a putative plastidial transit peptide. The expression analysis of truncated *Sultr4;1* fused with

jellyfish green fluorescent protein revealed localization in chloroplast membrane (Takahashi et al., 1999). However, expression of a full cDNA with GFP reporter gene localized the respective transporter in the tonoplast membrane (Takahashi et al., 2003).

After uptake from the soil, sulphate is either accumulated and stored in the vacuole or it is incorporated into organic compounds. ATP surphurylase (ATPS, AS) activates sulphate by converting it to adenosine-5'-phosphosulphate (APS). The enzyme has two forms: the major one is found in plastids, and a minor one is found in cytoplasm (Leustek, 2000). In plants, there are two distinct ways for further metabolisation of APS. The major pathway is the reductive assimilation, localized exclusively in plastids, the minor one – sulphation (Fig. 1-1). A variety of compounds such as polysaccharides (McCandless and Craigie, 1979), flavonoids (Varin et al., 1997), glucosinolates and jasmonates (Leustek, 2000) are the product of sulphation. Sulphation pathway is localized in cytoplasm, where APS is phosphorylated by APS kinase forming 3-phosphoadenosine-5'-phosphosulphate (PAPS). PAPS serves as a source of activated sulphate for specific sulphotransferases (ST's), which catalyse the covalent addition of sulphate to a variety of compounds (Bick and Leustek, 1998; Saito, 2004).

In plants the majority of sulphur is assimilated in the reduced form (Fig. 1-1). Eight electrons are required to reduce SO_4^{2-} to S^{2-}. First, APS is reduced to sulphite (SO_3^{2-}) by a GSH-dependent reductase called APS reductase (APR) [APS sulphotransferase], which transfers two electrons from reduced glutathione (Kopriva and Koprivova, 2004). Reduction of sulphite to sulphide (S^{2-}) is catalyzed by ferredoxin-dependent sulphite reductase, which realizes a transfer of six electrons from ferredoxin to sulphite. Thus, because of the huge need of electrons, sulphite reductase is localized in plastids of all tissues, where in photosynthetic cells, is supplied with electrons donated from photo system I (PS I) via ferredoxin. In non-photosynthetic tissues the electron supply comes from NADPH using ferredoxin-dependent $NADP^+$ reductase as an electron transmitter (Nakayama et al., 2000).

Synthesis of cysteine by condensation of the sulphide moiety and the carbon skeleton derived from serine *via O*-acetylserine (OAS) represents the final step in the reductive sulphate assimilation pathway. Serine acetyltransferase (SAT) generates an activated serine derivative, OAS, while OAS (thiol)-lyase (OASTL) transfers reduced sulphide to OAS in a β-replacement reaction that yields cysteine and acetate. Both enzymes form a dissociable complex, named cysteine synthase, which comprises a homotetramer of SAT and two dimers of OASTL (Saito, 2000; Hesse et al., 2004a, Hawkesford and De Kok, 2006). Cysteine synthase complexes have been demonstrated to be present in three compartments: cytosol, chloroplasts and mitochondria (Hoefgen et al., 2001; Saito, 2004; Hesse et al, 2004a). The respective activities of the cysteine synthase complex components are dependent upon their association state, which in turn is controlled by a free concentration of OAS. The current understanding of the complex formation is that OASTL bound to SAT is inactive in the synthesis of cysteine, but causes the stabilization of SAT while SAT is only active if bound to the complex. The OAS intermediate catalysed by the OASTL-bound SAT disrupts the enzyme complex and OASTL is released to convert OAS to cysteine (Hesse et al., 2004a). Moreover, at high levels of OAS (which occur under sulphur-limiting conditions), the SAT released from the complex becomes inactive, thus preventing further OAS synthesis. On the other hand, the sulphide accumulation

promotes complex formation, which turns the reaction back into the cysteine formation. Hence, reversible formation of the cysteine synthase complex becomes a sensor system and a regulatory centre of the reductive sulphur assimilation pathway (Hesse et al, 2004a; Saito, 2004; Hawkesford and De Kok, 2006).

1.1.3 Methionine synthesis and metabolism

Another sulphur-containing amino acid, methionine (Met) belongs to the aspartate family of amino acids. In plants, the branch point intermediate of threonine and methionine synthesis is *O*-phosphohomoserine (OPH, OPHS), which represents the common substrate for both threonine synthase (TS) and cystathionine gamma-synthase (CgS) (Fig. 1-1). OPH is either directly converted to threonine by TS or, in three-step mechanism, to methionine. The condensation of cysteine and OPH to form thioether cystathionine is the first committed step of *de novo* methionine biosynthesis. The reaction involves a *trans*-sulphuration process via a γ-replacement reaction. This step separates methionine synthesis from the other amino acids belonging to the aspartate family because of its connection with the sulphur assimilation pathway. Furthermore, the carbon precursor of methionine is distinct from that in yeast and bacteria (Hesse et al., 2004b). Cystathionine is subsequently converted by cystathionine β-lyase (CbL), which cleaves the C3 skeleton of cysteine leaving sulphur attached to the homoserine carbon skeleton and produces homocysteine. In the cytosol, homocysteine is methylated to form methionine. The reaction is catalyzed by methionine synthase (MS) using N5-methyltetrahydrofolate as a methyl-group donor. This activity is not only involved in *de novo* methionine synthesis but also in regeneration of the methyl group of S-adenosylmethionine (Matthews, 1999; Hesse and Hoefgen, 2003, Hesse et al., 2004b). Eventually, about 20% of methionine is incorporated into proteins while 80% is converted to SAM, which is *de facto* the end-product of the methionine biosynthesis pathway (Giovanelli et al., 1985).

1.1.4 SMM and SAM synthesis and metabolism

The formation of S-adenosylmethionine (SAM) from methionine and ATP is catalyzed by S-adenosylmethionine synthetase (Fig. 1-1). SAM synthetase is encoded by a gene family, which is highly conserved in all organisms. cDNA sequences indicated that these isoenzymes are located in the cytosol (Ravanel et al., 1998). Various SAM-dependent methylases catalyse transmethylation reactions, in which SAM is used as a methyl-group donor for the methylation of various receptors, such as amino acids, lipids, RNA and DNA (Droux et al., 2000). S-adenosylhomocysteine (SAHcy) produced in these reactions is a strong competitive inhibitor of SAM-dependent methylases, thus, it must be removed for proper functioning of these enzymes. This removal is ensured by SAHcy hydrolase that catalyses the hydrolysis of SAHcy to produce homocysteine and adenosine (Ravanel et al., 1998). In plants, SAM is involved in the synthesis of the ripening hormone ethylene (Kende, 1993) and in the synthesis of polyamines, such as spermidine and spermine (Walden et al., 1997). SAM decarboxylase converts SAM to decarboxylated SAM (dSAM), which is the rate-limiting step in the

polyamine biosynthesis pathway. Spermidine synthase catalyses the conversion of putrescine to spermidine, and spermine synthase regulates the conversion of spermidine to spermine. Both enzymes transfer an aminopropyl group from dSAM to their respective substrates. The synthesis of dSAM is inhibited by spermidine but increases in response to increasing putrescine. As both ethylene and polyamines are derived from SAM, the competition for SAM is possible (*Biochemistry and Molecular Biology of Plants*, 2000).

Furtermore, methylation of a methionine molecule at the level of the sulphonium group in the presence of SAM results in S-methylmethionine (SMM) formation that has been found occurring commonly in plants (Mudd and Datko, 1990). SMM is considered as a storage compound for two methyl groups, one C4 molecule and one S atom. Indeed, it can act as a methyl donor in a reaction with homocysteine to form two molecules of methionine (Fig 1-1). The synthesis and utilisation of SMM is considered to be balanced in time or in space to make the overall sequence a contribution in the net methionine synthesis (Giovanelli, 1990).

1.1.5 Sulphate regulation of transport and metabolism

1.1.5.1 Development

Depending on the developmental stage and function, the different tissue and organs differ in their demand for sulphur. The sulphur assimilation enzymes are highly active in young leaves and root tips, and decline markedly in older tissues. The activity and level of chloroplast ATP sulphurylase and APS reductase was found to decline approximately 3-fold as the leaves aged. Such a developmental expression pattern suggests that sulphate assimilation is highly active in growing tissues where there is a high demand for cysteine and methionine for protein synthesis (Leustek, 2002; Hawkesford and De Kok, 2006).

1.1.5.2 Reductant supply

Sulphur assimilation does not appear to be strongly regulated by light, however photosynthesis has an influence on the process. Sulphite reduction in chloroplasts clearly depends on reduced ferredoxin produced from the photosynthetic light reactions, but sulphite reduction also occurs in nonphotosynthetic plastids, where the reductand source is most likely generated by glycolysis and the pentose phosphate pathway (Leustek, 2002). However, sulphate assimilation in root plastids is a minor contributor to the reduced sulphur pool in plants; primary source is the shulphate assimilated in shoot chloroplasts (Hawkesford and De Kok, 2006). As many nuclear-encoded genes for plastidic localized enzymes, APS reductase activity and flux through the sulphate assimilation pathway are induced by light (Kopriva et al., 1999).

Unlike the enzymes participating in nitrate and carbon assimilation, the sulphur assimilation enzymes are far less diurnally regulated. The sulphur assimilation genes showing the most pronounced circadian changes in mRNA level include: *Sultr3;1* and *Sultr2;2*, two sulphate transporters

expressed primarily in leaf, *APR2* APS reductase, *SAT-p*, the plastid localized form of serine acetyltransferase, and a phosphoglycerate oxidase gene encoding the first enzyme of serine synthesis. In *Arabidopsis*, mRNA of APR starts to accumulate 4 h before light onset, indicating that the enzyme is not controlled by light alone, but also by an internal signal. Indeed, feeding of sucrose in the dark led to increased levels of APR mRNA, protein and enzyme activity in roots (Kopriva et al., 1999). Interestingly, *APR2* mRNA was more susceptible to regulation by light/dark cycles and sucrose feeding than *APR1* and *APR3* mRNA (Kopriva et al., 1999). On the other hand, other genes like i.e. sulphite reductase does not show the circadian phasing (Leustek, 2002). The circadian phasing of sulphate transporters and enzymes for sulphate assimilation before the onset of the light period are thought to ensure that sulphate and the machinery for its conversion into cysteine is present in chloroplasts at a time just prior to the time when reducing power is generated by photosynthesis (Kopriva et al., 1999; Harmer et al., 2000).

1.1.5.3 Stress induced demand for sulphate

The rate of the cysteine synthesis depends on a number of factors, such as nutritional and environmental conditions and stresses. In particular, oxidative stress, toxins and heavy metals induce a greater demand for gluthatione synthesis, which in turn increases the demand for cysteine (Leustek, 2002; Saito, 2004).

External sulphate availability has the most significant impact on the sulphur assimilation rate. Plants that are grown with insufficient levels of sulphate develop symptoms of sulphur deficiency, which include chlorosis of young leaves, growth retardation and altered root morphology, which is thought to be related with changes in root auxin metabolism (Hawkesford, 2000; Nikiforova et al., 2003). Responses to insufficient sulphur availability to match demand may be graded into initial responses specific for sulphur nutrition, followed by more complex responses which may be less specific and invoked by other nutrient deficiencies (Hawkesford and De Kok, 2006). A hypothetical sequence of responses occurring upon limitation of S-supply is schematically depicted in figure1-2.

Molecular mechanisms for the responses to sulphur deprivation in higher plants were recently investigated by using transcriptome approaches (Nikiforova et al., 2003; Hirai et al., 2003; Maruyama-Nakashita et al., 2003). It is well known that sulphate uptake and assimilation activities are derepressed under sulphur-deficient conditions. In *Arabidopsis*, this derepression is correlated with the inducible expression of a particular set of genes that encode i.e. sulphate transporters, especially the high affinity isoforms, *Sultr1;1*, *Sultr 1;2* but also *Sultr2;1*. Other lower-affinity forms are unresponsive or respond more slowly to changes in external SO_4^{2-} supply. APS reductase and the chloroplast localized serine acetyltransferase expression is increased by sulphur starvation and is repressed by treating plants with reduced forms of sulphur (Saito, 2000; Leustek, 2002). Moreover, APS was found to be up-regulated by sulphur deficiency not only expressionally but also at the protein and activity level (Takahashi et al., 1997; Bolchi et al., 1999; Lappartient et al., 1999).

Controlling the sulphur reduction pathway by regulation of APS reductase makes intuitive sense: toxic sulphite and sulphide must not be allowed to accumulate. ATP sulphurylase, which

produces the substrate for the sulphation pathway, would be a poorer target for regulation than APR because the ability of the former to function in the forward direction is strongly dependent on the removal of APS by APS reductase or APS kinase (*Biochemistry and Molecular Biology of Plants*, 2000). Unlike the APS reductase, the steady-state concentration of the mRNA for sulphite reductase in plants remains constant in response to sulphur starvation or to feeding of reduced sulphur compounds, and the activity of sulphite reductase is not affected appreciably by sulphur nutrition. Again, because of sulphite toxicity, especially if accumulated, presumably sulphite reductase activity is maintained in excess of the preceding enzyme in the pathway, APS reductase (*Biochemistry and Molecular Biology of Plants*, 2000).

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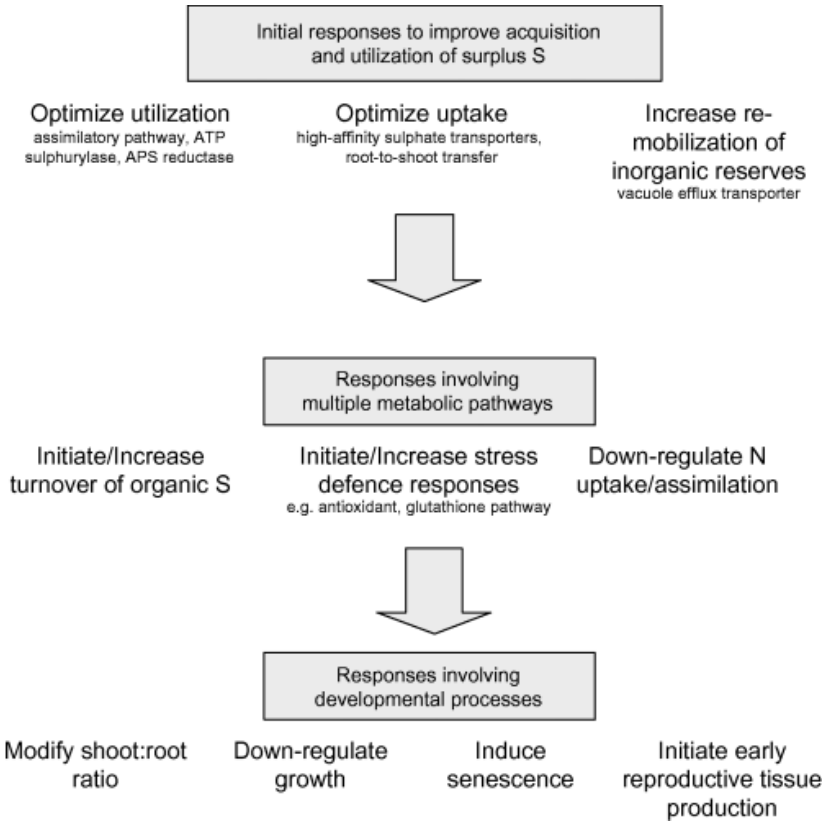


Fig 1-2 A hypothetical sequence of responses occurring upon limitation of sulphur supply to a flowering plant (taken from Hawkesford and De Kok, 2006). APS, adenosine phosphosulphate.

The short-term effect on expression of sulphate transporters, APR and chloroplast SAT genes is observed primarily in roots (Takahashi et al. 1997; Smith et al., 1997). The level of mRNA and protein for other enzymes of reductive sulphate assimilation, such as ATP sulphurylase and OAS thiollyase, increase only after long-term sulphur starvation. Chronic sulphate starvation is also necessary to induce expression of sulphate assimilation proteins in leaves (Lappartient et al., 1996; 1999; Lee, 1999). The delayed response in leaves, compared with roots, which is especially characteristic for mature plants, could be due to the large store of sulphate in the vacuoles of leaf cells, which may buffer them against short-term reductions in external sulphate concentration.

With a limited input of sulphur, levels of sulphur-containing metabolites such as cysteine and gluthatione decrease, while the precursors, serine and OAS, accumulate (Nikiforova et al., 2003). On

the other hand, when plants grow in an S rich environment, downstream metabolites repress transcription of many genes involved in the sulphur assimilatory pathway.

Glutathione and cysteine are thought to be negative regulators of the reductive sulphur assimilation process, as high concentrations of Cys and GSH repress activities of key enzymes in the S assimilatory pathway (Leustek and Saito, 1999; Saito, 2000, Leustek, 2002). Administration of high concentrations of cysteine and glutathione to plant roots leads to lowered steady-state levels of mRNAs for the SO_4^{2-} transporters, ATP sulphurylase, and APS reductase (Bolchi et al., 1999; Lappartient et al., 1999). Glutathione, which is known to be transported through the phloem sap, is markedly reduced after short-term sulphur starvation and therefore may be a signaling molecule that represses the activity of genes encoding key enzymes in the S assimilation pathway (Leustek and Saito, 1999; Saito, 2000; Grossman and Takahashi, 2001).

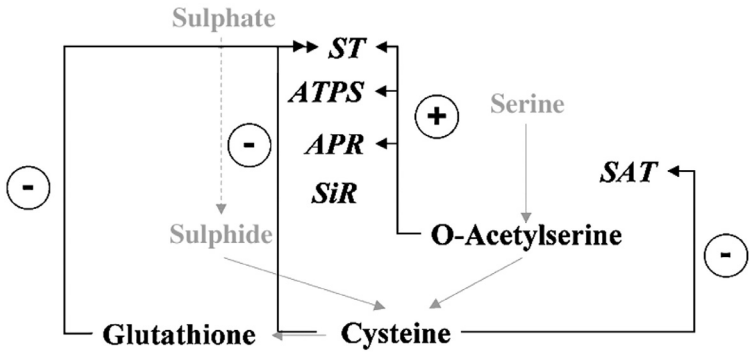


Fig 1-3 Metabolic control of sulphate uptake and assimilation (taken from Hesse etal., 2004a). Solid lines in grey represent metabolite fluxes; black lines are feedback control loops. ST, sulphate transporter; ATPS, ATP sulphurylase; APR, APS reductase; SiR, sulphite reductase; OASTL, O-acetylserine (thiol)lyase; SAT, serine acetyltransferase.

OAS does not only serve as the carbon backbone for cysteine formation, but is also a positive regulator of sulphate uptake and assimilation (Hesse et al., 2004a). Exogenous supply of OAS leads to an increased thiol content indicating that the supply of OAS may limit cysteine synthesis in the presence of sulphate, as shown by overexpression of serine acetyltransferase resulting in increased levels of cysteine and glutathione in transgenic tobacco and potato plants (Blaszczyk et al., 1999; Harms et al., 2000; Wirtz and Hell, 2003). On the other hand, OAS, which accumulates during sulphur starvation (Kim et al., 1999; Awazuhara et al., 2000; Nikiforova et al., 2003) acts positively on the transcript and activity levels of sulphate transporters, ATP sulphurylase, APR, sulphite reductase, plastidial OASTL, and cystosolic SAT, as shown in OAS-feeding experiments (Clarkson et al., 1999; Bolchi et al., 1999; Koprivova et al., 2000; Saito, 2000; Hawkesford, 2003). Based on these results, Hawkesford (2000) proposed a model in which the expression of genes involved in uptake and assimilation are under positive regulation by OAS. A series of feedback loops are proposed in which cellular concentrations of pathway intermediates may act as part of a signal perception and transduction pathway to repress or activate expression of genes encoding the proteins controlling some of the individual steps in the pathway (Fig. 1-3). This model might reflect the metabolic regulation on a cellular level but not on a plant level. OAS fed to roots revealed that, for example, APR

responded only locally to the inducer and not in other tissues (Hesse et al., 2003). Additionally, OAS participates in an allosteric feedback regulation of SAT, by causing reversible formation of the cysteine synthase complex, as discussed before (see chapter 1.1.2).

1.1.5.4 Sulphur/nitrogen balance

Since cysteine is the first committed molecule in plant metabolism containing both sulphur and nitrogen, plants ability to compensate for sulphur and nitrogen deficiencies is for cysteine biosynthesis critically important. Nutrients such as nitrate and sulphate (and carbon) act as signals; they trigger molecular mechanisms that modify biosynthetic pathways and thereby have a profound impact on metabolite fluxes. Cysteine biosynthesis is modified by regulators acting at the site of uptake and throughout the plant system (Hesse et al., 2004a).

The regulatory interaction between sulphate assimilation and nitrate reduction is believed to occur at the transcriptional level (Prosser et al., 2001). Nitrate itself can induce genes of sulphate uptake and assimilation and, thereby, may increase sulphate assimilation rate or capacity. Two putative sulphate transporter genes and an APR gene are known to be induced by nitrate in roots. On the other hand, the absence of nitrate can down-regulate the capture and assimilation of sulphate by the roots. In various systems APR expression and activity was shown to be down-regulated by nitrogen starvation (Yamaguchi et al., 1999; Koprivova et al., 2000). Among the genes involved in sulphur metabolism, one encoding a SAT is known to be induced by nitrate in shoots. This means that under nitrate re-supply leaf cells can synthesize OAS at high rates to ensure the amino acid balance under increased nitrogen-assimilation. Hence, it can be speculated that in leaves, cysteine synthesis is impaired under nitrogen deficiency (Hesse at al., 2004a).

Although deprivation of nitrogen leads to a disruption of sulphur metabolism, the effect of sulphur depletion on nitrogen metabolism is much less evident. In roots, sulphate depletion did not significantly affect nitrate uptake and assimilation when it occurs for a short period. Longer sulphate starvation stimulates the accumulation of glutamine as a nitrogen store probably because of limited protein synthesis under sulphur deficiency (Hesse at al., 2004a). Reduced protein synthesis is accompanied by the accumulation of organic and inorganic nitrogenous compounds. Plants starved of sulphur accumulate arginine and asparagine with reduced levels of sulphur-containing amino acids such as cysteine and methionine. (Thomas et al., 2000; Prosser et al., 2001; McCallum et al., 2002; Nikiforova et al., 2003; 2005b).

Although the interrelationship of sulphur and nitrogen metabolism seems to be of a hierarchical nature, that of nitrogen having priority over that of sulphur, sulphur interacts with nitrogen in such a way that lack of one reduces the uptake and assimilation of the other (Hesse at al., 2004a). The response to sulphur starvation is attenuated if plants are at the same time also limited for nitrogen. Nitrogen limitation blocks the accumulation of transcripts for ATP sulphurylase and APS reductase normally induced by sulphur limitation of Arabidopsis (Yamaguchi et al., 1999; Lee 2000; Koprivova et al., 2000). This result suggests that some nitrogen-containing compound is necessary for de-repression of sulphur assimilation genes.

Indeed, sulphur uptake and assimilation has been shown to be dependent upon the constant supply of the precursor of cysteine, O-acetylserine, which, in turn, is dependent upon adequate nitrogen and carbon availability (Koprivova et al., 2000; Kopriva et al., 2002). OAS thus links assimilatory sulphate reduction with carbohydrate and nitrogen metabolism and has been proposed as a signaling molecule coordinating these three pathways (Brunold, 1993). Transcriptome profiling studies have suggested that OAS is a global regulator of large numbers of genes, specifically in many cases the same genes that are regulated by sulphurnutritional status (Hirai et al. 2003). This is consistent with OAS mimicking sulphur limitation as it creates an increased demand for reduced sulphur. All of these data remain consistent with the idea that OAS accumulation reflects an imbalance of nitrogen and sulphur nutrition, rather than an early metabolic signal which the plant can use to fine-tune these pathways (Hawkesford and De Kok, 2006).

1.1.5.5 Methionine regulation

Several lines of recent evidence show that the branch point between cystathionine γ-synthase (CgS) and threonine synthase (TS) plays a major regulatory role in the flux of carbon into methionine, and that CgS competes fairly weakly with TS for their common substrate *O*-phosphohomoserine (OPH) (Bartlem et al., 2000; Gakiere et al., 2000; Zeh et al., 2001). The enzymatic activity of plant TS is strongly stimulated by SAM, the end-product of the competing pathway (Fig. 1-4). Because K_m values of fully activated TS for OPH have been shown to be 250–500-fold lower than those of CgS, carbon flux is directed into the threonine branch when methionine and, hence, SAM levels are high (Curien et al., 1998; Ravanel et al., 1998; Hesse and Hoefgen, 2003; Hesse et al., 2004b).

Recent results from transgenic *Arabidopsis* plants manipulated in CgS enzymatic activity levels gave rise to the hypothesis that CgS exerts major flux control for methionine metabolism in *Arabidopsis* (Gakiere et al., 2000, 2002; Kim and Leustek, 2000; Kim et al., 2002). This hypothesis is supported by studies indicating that *Arabidopsis* CgS is feedback-regulated by methionine itself or derivatives at the post-transcriptional level (Fig. 1-4). Molecular investigations of CgS regulation suggest that a stretch of 39 amino acids, encoded by exon1 of *AtCgS* and designated as the MTO1 region, act in *cis* to destabilize its own transcript, the *CgS* mRNA, in response to high levels of methionine or related metabolites such as SAM (Chiba et al., 1999, 2003; Bartlem et al., 2000; Suzuki et al., 2001; Lambein et al., 2003). Accordingly, *AtCgS* mRNA levels and enzymatic activities are reduced in the presence of excess methionine in *Arabidopsis* (Chiba et al., 1999; Bartlem et al., 2000). The regulatory mechanism is not known, but computer analysis predicts that mRNA sequences near the MTO1 region can form stable stem-loop structures (Amir et al., 2002), supporting a model of post-transcriptional control by this region (Chiba et al., 1999; Kim et al., 2002; Lambein et al., 2003). In this model it is proposed that the regulation occurs during translation when the nascent polypeptide of CgS and its mRNA are in close proximity. This model predicts that inhibition of translation abolishes the regulation (Hesse et al. 2004b). Described above post-transcriptional regulation machinery seems to be specific for certain species; it was documented for *Arabidopsis thaliana* and *Lemna paucicostata*, however, it might not exist in potato. It was shown that increasing the soluble Met pool in potato leaves

was not accompanied by changes in levels of CgS transcript or activity (Kreft et al., 2003). Thus, directing carbon flow either to methionine or threonine biosynthesis seems to be controlled in potato solely by the activation of TS enzyme activity in the case of SAM accumulation (Hesse and Hoefgen, 2003; Hesse et al., 2004b).

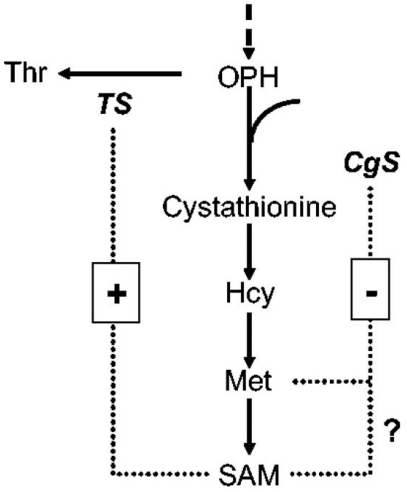


Fig 1-4 The model of methionine biosynthesis control of *Arabidopsis thaliana* and *Lemna paucicostata* (taken from Hesse et al., 2004b). TS, threonine synthase; CgS, cystathionine γ-synthase; Thr, threonine; OPH, O-phosphohomoserine; Hcy, homocysteine; Met, methionine; SAM, S-adenosyl methionine.

1.2 Transcriptional control

1.2.1 Sulphate signalling and transcriptional regulation in lower organisms

Sulphur sensing, signalling and transcriptional regulation has been an intensively studied topic, especially in lower organisms, such as algae, yeast and bacteria.

1.2.1.1 Algae

When placed in a sulphur-deficient environment, the green algae *Chlamydomonas reinhardtii* responds by inducing specific and general responses to sulphur deficiency. General responses include a decline in photosynthetic activity and cell division (Davies et al., 1996) and an increase in starch accumulation (Ball et al., 1990). Specific responses include the induction of a set of periplasmic proteins including an extracellular arylsulphatase (which is a periplasmic protein specifically synthesized by *C. reinhardtii* during sulphur limitation catalyzing the hydrolysis of soluble arylsulphate esters in the medium and thereby releasing free SO_4^{2-} for assimilation by the cell (de Hostos et al., 1988), an increase in sulphate transport activity (Yildiz et al., 1994), and elevated expression of ATP sulphurylase (Yildiz et al., 1996), APS reductase, SAT and OAS (thiol)lyase (Ravina et al., 1999).

Expression of sulphur-related genes in *C. reinhardtii* is known to be controlled by at least three specific proteins, encoded by *SAC1* (Sulphur-ACclimation), *SAC2*, and *SAC3* genes, which were

identified in mutants defective in regulating expression of arylsulphatase activity (Davies et al., 1994). sac*1* and *sac2* mutants are deficient in synthesizing arylsulphatase, whereas *sac3* constitutively expresses arylsulphatase in sulphate-replete medium. All three mutants are unable to increase sulphate transport to the same extent as wild-type cells when deprived of sulphate. In addition, *sac1* but not *sac2* or *sac3*, is unable to down-regulate photosynthesis in response to sulphur starvation (Davies et al., 1996). Thus, *sac1* is deficient in the general and specific responses to sulphur starvation, whereas *sac2* and *sac3* appear to be deficient in only the specific responses (Ravina et al., 2002).

Most responses of *Chlamydomonas* to S limitation require the SAC1 protein. *SAC1* gene encodes a polypeptide predicted to have 10 transmembrane domains. This protein is similar to ion transporters from a number of different organisms, with the greatest degree of similarity to a Na^+/SO_4^{2-} transporter from rat kidneys. Although it is possible that SAC1 can function in the uptake of sulphate, the phenotype of the *sac1* mutant strongly suggests that it plays an important role in regulating cellular responses to S deprivation (Davies et al. 1996). It may be involved in sensing the level of sulphate in the medium (Davies and Grossman, 1998). While the signaling mechanism used by SAC1 has not been established, SAC1 is predicted to have a large intracellular loop, located between transmembrane helices 4 and 5, with two TrkA domains that have been suggested to bind NAD^+ or another unidentified ligand (Schlosser et al. 1993; Anantharaman et al. 2001). Thus, this region of the protein has the potential to function in signaling as a consequence of interactions with an intermediate metabolite and/or specific proteins (Pollock et al. 2005).

The *SAC2* gene has not been cloned, but the phenotype of the *sac2* mutant suggests that it is either directly or indirectly involved in the transcriptional regulation of arylsulphatase genes and possibly other genes induced during sulphur limitation (Davies et al., 1994). The sulphate-deprived *sac2* mutant accumulates APS reductase transcripts, but not APS reductase activity, suggesting that *SAC2* is needed for posttranscriptional control of APS reductase activity in cells deprived of sulphate (Ravina et al., 2002).

sac3 mutant was selected for low-level constitutive arylsulphatase activity (high level of Ars activity when grown on –S medium and a low level of Ars when grown on +S medium). In addition, under S-deprivation, SO_4^{2-} transport could not be activated to the same extent as in wild-type cells. These results suggest that *SAC3* encodes a negative regulator of *ARS* gene activity and a positive regulator of gene(s) encoding the SO_4^{2-} transport system (Davies at all., 1994; 1999). *SAC3* encodes a putative Ser-Thr protein kinase; the exact mechanism by which this kinase modulates the transcription of genes in both a positive and negative sense requires additional analyses (Grossman and Takahashi, 2001; Pollock et al., 2005).

A speculative model of S-dependent signal transduction operating in *C. reinhardtii* was proposed by Pollock et al. (2005). In this model, SAC1 is likely the SO_4^{2-} sensor of the cell. It may function as a transporter (although this has not been established), and at the same time monitor the SO_4^{2-} concentration in the environment surrounding the cell. In S-sufficient conditions (Fig. 1-5a), signaling from SAC1 may occur through ARS11 (a protein kinase homologous) to the SAC3 polypeptide. SAC3 freely phosphorylates Y, an unidentified peptide, to repress the transcription of

S-responsive promoters, however, this phosphorylation may not depend on SAC1 or ARS11. Furthermore, under these conditions the transcription factor X (not yet identified) is maintained in an inactive state, either because it cannot be phosphorylated by ARS11 and/or a phosphatase is actively removing the phosphate group from this putative regulator. When the cells experience S deprivation (Fig. 1-5b), SAC1 signals, with the binding of a cyclic nucleotide monophosphate, through ARS11 to de-repress the SO_4^{2-} responsive promoters, and through SAC3 to activate the SO_4^{2-} transporters, possibly by causing phosphorylation of a Thr residue in the STAS domain at the C-terminal end of the transporters. ARS11 may directly or indirectly phosphorylate the transcription factor X, which positively regulates many of the S-stress associated genes. Phosphatases may help maintain SAC3 and ARS11 in a dephosphorylated state. In sum, it is suggested that a SAC1 complex initiates an S-status-dependent phosphorelay that controls the acclimation of the cells to S deprivation through the regulation of both transcriptional and post-transcriptional processes. Currently, no experimental evidence has shown that protein– protein interactions occur between these regulators.

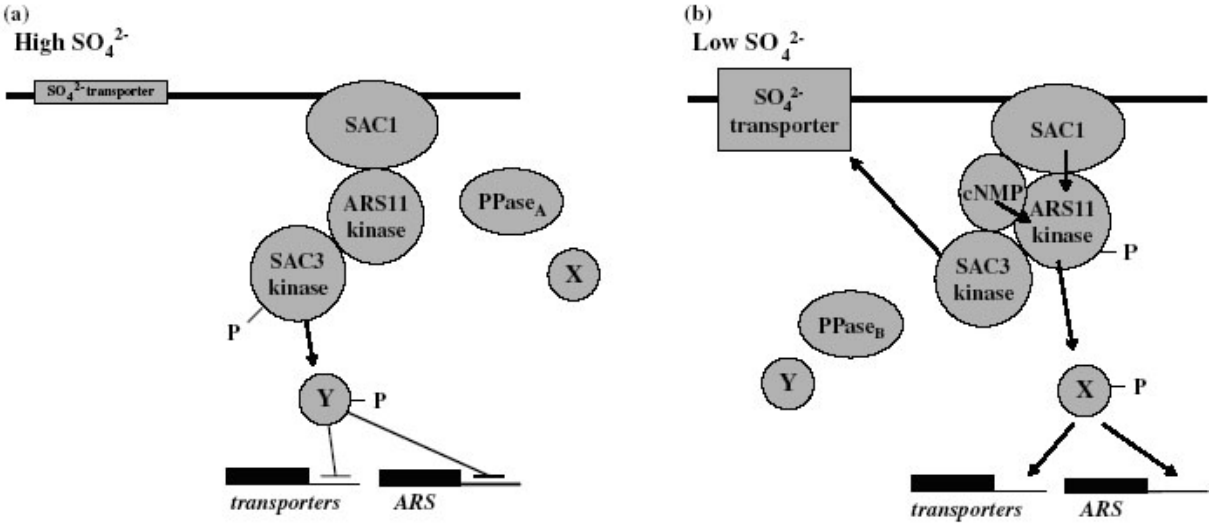


Fig 1-5 Speculative model of S-dependent signal transduction operating in C. reinhardtii (taken from Pollock et al., 2005). The model depicts the activity of the signal transduction cascade in S-replete **(a)** and S-depleted **(b)** environments. cNMP, cyclic nucleotide monophosphate; PPase, protein phosphatase.

Interestingly, the deduced polypeptide sequence of SAC1 and the phenotype of the *sac1* mutant display some similarities with the sequence of *Snf3* of *Saccharomyces cerevisiae* and the phenotype of the *snf3* mutant, respectively. *Snf3* has strong sequence similarity to a glucose transporter, but is a yeast regulatory protein that governs the expression of genes involved in hexose utilization and thereby functions in the acclimation of the cells to the glucose status in the medium (Pollock et al., 2005). The sequence similarity between SAC1 and Snf3, as well as between SAC3 and yeast Snf1, raises the possibility that polypeptides whose original function was to bind and transport various substrates into cells may have evolved into regulatory elements (Grossman and Takahashi, 2001; Pollock et al., 2005).

1.2.1.2 Fungi

Biosynthesis of sulphur amino acid in yeast has long been known to be specifically and negatively regulated. Synthesis of the enzymes required for sulphate assimilation, as well as methionine and cysteine synthesis, is repressed by the addition of methionine or SAM (AdoMet) to the growth medium (Cherest et al., 1969). Typically, wild-type cells grown in the presence of 1mM L-methionine (repressive conditions) express less than 10% of the enzymatic activities measured after growth in ≤0,05 mM methionine (nonrepressive conditions). In contrast, addition of high concentrations of either homocysteine or cysteine does not significantly affect the synthesis of these enzymes.

Molecular cloning and analysis of structural genes subjected to SAM-mediated negative regulation demonstrated that their expression is transcriptionally regulated (Cherest et al., 1985; Sangsoda et al., 1985). Analysis of yeast homocysteine synthase gene (*MET25*) identified two important regions in its upstream region. The first, centered around CACGTG palindromes, was required for de-repression of sulphur-related gene expression when intracellular SAM concentrations are low. This *cis*-acting element is the binding site for the heteromeric transcription activation complex, Cbf1-Met4-Met28 (Kuras et al., 1996; 1997). The second functional sequence is the consensus sequence AAANTGTG. This element was first characterized as being required for full repression of *MET25* at high intracellular SAM concentrations. This DNA sequence was subsequently shown to bind two specific factors, Met31 and Met32, whose functions are not yet fully understood (Blaiseau et al., 1997).

Cbf1p consists of 351 amino acids with a calculated molecular weight of 40,000, and contains a basic helix-loop-helix (bHLH) domain, which was proven to be required for binding to the TCACGTG sequence (Cai and Davies, 1990; Mellor et al., 1990; 1991). Structural analysis of the carboxyterminal domain of Cbf1 predicted some resemblance to a leucine repeat, showing that it shared functional characteristics with the bHLH-LZ DNA binding factors (Dowell et al., 1992). The analysis of *cbf1* mutants supported the proposed implication of Cbf1 in chromosome segregation and transcriptional activation of sulphur metabolism genes. *CBF1* disruption strongly decreases PAPS reductase (*MET16*) transcription when cells are grown under nonrepressive conditions. Moreover, these cells lack sulphate permease activity. However, inactivation of the *CBF1* gene does not impair the transcription of all genes of the sulphate assimilation pathway to the same extent. While the α-subunit of sulphite reductase (*MET10*), APS kinase (*MET14*), and PAPS reductase (*MET16*) gene expression was shown to depend strictly on the presence of active Cbf1p, ATP sulphurylase (*MET3*) and homocysteine synthase (*MET25*) transcription reached half of the wild-type levels in a *cbf1* mutant (Kuras and Thomas 1995a; Thomas et al., 1992a).

Physiological analysis of methionine auxotrophs identified mutations at the *MET4* locus that rendered cells selenate resistant and unable to grow on inorganic sulphur sources or on cysteine (Thomas et al., 1992b). Enzyme assays on *met4* cell extracts revealed undetectable levels of all enzymes required for sulphate assimilation (Thomas et al., 1992a). *Met4* mutations also impaired sulphate transport. As expected from the enzyme assays, Northern experiments demonstrated that transcriptional activation of homoserine trans-acetylase (*MET2*), *MET3*, both subunits of sulphite

reductase (*MET5* and *MET10*), *MET14*, *MET16*, and *MET25* genes does not occur in *met4* mutants. Thus, Met4 is considered as a main, strong transcriptional activator which function is inhibited by increases in the intracellular SAM concentration (Thomas et al., 1990; 1992a; Aranda and del Olmo, 2004). *MET4* encodes a rather large protein of 666 amino acid residues. Sequence analysis revealed that Met4 contains a basic leucine zipper motif (bZIP) and an inhibitory region, which interacts with Met30 regulatory protein (Thomas et al. 1992a).

Met30 is devoid of canonical DNA binding motifs but contain five 40-amino-acid WD40 motifs and F-box motive (Thomas et al., 1995; Bai et al., 1996). Met30 was found to interact via its F-box motive with two components of SCF complex, Skp1 and Cdc53. SCF complexes belong to the group of E3 ubiquitin-protein ligases. The F-box motive is an important modular subunit of SCF complexes, as it provides them with substrate specificity (Sizemore and Paietta, 2002). There are evidences that the Met4 regulation by Met30 involves proteolysis of Met4 protein (Kuras and Thomas, 1995b). SCF complex containing the Met30 as the F-box constituent was found to be involved in the repression of the homocysteine synthase gene (*MET25*) (Patton et al., 1998; Smothers et al., 2000).

Met28, a second bZIP factor, is required for full induction of *MET* genes (Kuras et al., 1996). A *met28* chromosomal deletion lowers maximal *MET3*, *MET10*, *MET14*, and *MET16* transcription compared to the wild type. By contrast, *MET25* gene transcription remained unaffected by the *met28* mutation. Met28 therefore appears to function as a positive effector of transcription of several *MET* genes when the intracellular SAM concentration is low.

By using the two hybrid system it was determined which binary protein-protein contacts allow assembly of the Cbf1-Met4-Met28 complex. Met4 and Met28 were shown to interact through their respective leucine zipper domains, while Met4 and Cbf1 were shown to be in contact through the bZIP domain of Met4 and the bHLH domain of Cbf1. No direct interaction between Met28 and Cbf1 could be detected by such a method (Kuras et al., 1996; Fig. 1-6a). The Cbf1-Met4-Met28 complex exhibits several distinctive features. It associates one multifunctional factor (Cbf1) with two specific factors (Met4 and Met28). Cbf1, like other bHLH proteins, binds to DNA as a dimer and tether the specific transcription activator Met4 to the promoter. Met28 contributes to the transcriptional regulation of the sulphur amino acid pathway by performing two functions. First, it is an indispensable architectural component of the Cbf1-Met4-Met28 complex, and the interactions between its leucine zipper domain and that of Met4 are critical for the formation of this complex. Second, its stimulation of the DNA binding activity of Cbf1 increases the DNA binding affinity of the complex (Thomas and Surdin-Kerjan, 1997).

Following model was proposed to explain the SAM regulation of Met4 activity (Fig 1-6b). At high intracellular SAM concentrations, the inhibitory region of Met4 protein interacts with a regulatory protein, Met30, which prevents the activation domain of Met4 from contacting the basal transcription apparatus, thereby resulting in low levels of transcription activation. At low SAM concentrations, the auxiliary domain helps the Met4 inhibitory region to dissociate from the Met30 regulatory protein, thereby freeing the activation domain to function (Kuras and Thomas, 1995b; Thomas and Surdin-Kerjan, 1997).

Interestingly, no regulation specifically affecting the expression of *CBF1* has been reported. The transcription of both *CBF1* and *MET4* was found to be independent of the intracellular level of SAM. In contrast, transcription of the *MET28* gene was demonstrated to be regulated by increases in intracellular levels of SAM (Kuras et al., 1997). Northern blot experiments revealed that the addition of 1mM L-methionine to the growth medium resulted in a rapid cessation of *MET28* transcription. The *MET28* transcripts were shown to be very unstable, having a half-life of less than 5 min. Additional Northern blot experiments revealed that *MET28* transcription depends on the presence of functional Met4. It was further demonstrated that both Cbf1 and Met28 are involved in the transcriptional activation of the *MET28* gene. The biochemical activity of Met28, which allows it to stimulate the Cbf1 DNA binding activity, and the mechanisms underlying the transcriptional control of the *MET28* gene demonstrate the existence of a positive regulatory loop within the sulphur network (Kuras et al., 1997).

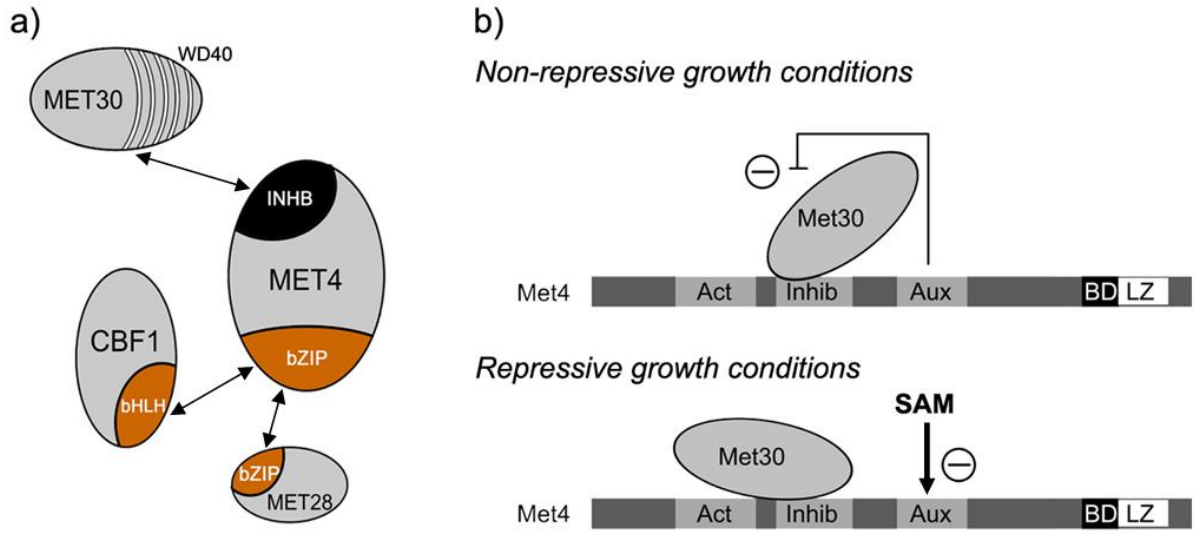


Fig 1-6 Regulation of the expression of the *MET* genes is *Saccharomyces cerevisiae*. Interaction between the different factors **(a)** and model for the SAM (Ado Met) regulation of Met4 protein activation function **(b)** (redrawn from Thomas and Surdin-Kerjan, 1997). Act, activation domain; Inhib, inhibitory region; Aux, auxiliary domain; BD, basic domain; LZ, leucine zipper.

The sulphur regulatory system of *Neurospora crassa* consists of a group of sulphur-regulated structural genes (e.g., arylsulphatase, sulphate permease) that are under coordinate control of the CYS3 positive regulator and Sulphur CONtroller (SCON) negative regulators (Sizemore and Paietta, 2002). CYS3 consists of 236 amino acids and, like the yeast Met4 and Met28, is a bZIP protein (Fu et al., 1989). However, unlike Met4 and Met28, CYS3 binds by itself to the upstream regions of coregulated genes. Mutants of the *CYS-3* gene are unable to induce transcription of the genes encoding ARS and sulphate permease (Paietta, 1995; Marzluf, 1997). *CYS-3* gene expression is controlled in part by a positive feedback loop involving the CYS3 protein and in part by the negative regulatory sulphur controller genes, *scon-1* and *scon-2* (Paietta, 1990; 1992). *Scon-1* and *scon-2* mutants show constitutive expression of *CYS-3* and the sulphur structural genes. *Scon-2* has been cloned and found to be expressed only under conditions of sulphur limitation. Like Met30 in yeast, the SCON2 protein contains two domains implicated in protein-protein interactions: a region of six WD40 repeats and an F-Box motif, which was found to be responsible for interaction with newly isolated

SCON-3 regulator (Kumar and Paietta, 1995). A SCON-2/SCON-3 complex regulates then the *CYS-3* expression (Sizemore and Paietta, 2002).

MetR protein isolated from *Aspergillus nidulans* has similar functions in sulphur regulation as the CYS3 proteins from *N. crassa* (Natorff et al., 2003). MetR is a member of bZIP proteins family of DNA-binding proteins and was reported to function as a transcriptional activator of several sulphur-related genes encoding i.e. a sulphur controller, sulphate permease, ATP-sulphurylase, homocysteine synthase and cysteine synthase (Paszewski et al., 2000; Natorff et all., 2003). However, similarly to the *S. cerevisiae MET4* gene (Mountain et al., 1993), transcription of the *Aspergillus metR* gene is not regulated by sulphur source. MetR protein is not indispensable for the transcription of its own gene. In contrast to that, transcription of the *Neurospora cys-3* gene is strongly repressed in the presence of high methionine (repressing conditions) (Natorff et al., 2003). *A. nidulans metR1* mutants show an increased level of sconC transcript under sulphur derepressing conditions, which indicate that MetR may act as a negative regulator of *sconC* expression (Piotrowska et al., 2000). SCONC, a homologue of Skp1, and the F-box containing SCONB, homologue of Met30, were identified and characterised as a two *A. nidulans* genes encoding homologues of yeast SCF ubiquitin ligase subunits (Natorff et al., 1998; Piotrowska et al., 2000). SCONB and SCONC proteins interact with themselves and are involved in the sulphur metabolite repression system, which shuts off the sulphate assimilation pathway under repressing conditions.

1.2.1.3 Bacteria

Regulation of methionine biosynthesis in *Escherichia coli* involves a complex of the MetJ aporepressor protein and S-adenosylmethionine (SAM) repressing expression of most genes in the *met* regulon (La Monte and Hughes, 2006; Augustus et al., 2006). MetJ is a 12 kDa protein that is reported to form a homodimer in its native state. It was the first structurally characterized member of the RHH (ribbon–helix–helix) class of DNA-binding proteins that interact with DNA bases via a pair of *β*-strands (Marincs et al., 2006; Fig. 1-7a). Repressor activity results from MetJ binding to specific 8-bp DNA sequences, called metboxes, located in the promoter regions of genes in the *met* regulon. Although MetJ selectively binds metbox sequences alone, its affinity for metbox DNA is enhanced several fold by its co-repressor, SAM, an end-product of methionine biosynthesis (Augustus et al., 2006).

Multiple MetJ dimers bind to operator sequences that contain two to five contiguous metboxes. A minimum of two tandem metboxes are required for efficient MetJ binding *in vitro* and repression of transcription *in vivo* (Phillips et al., 1989). Adjacent MetJ dimers can interact with each other when they are bound to tandem metbox DNA sites, making assembly of higher order repressor complexes a cooperative process (He et al., 1992). There is a great deal of sequence variability among metboxes within each operator, but the shared consensus sequence is palindromic 5'-AGACGTCT-3' (Phillips et al., 1989)

Genes most induced by defects in *metJ* gene include *metA, B, C, E, F, K, glyA* (as the downstream gene in the operon) and *metR* (encoding another regulatory protein that, by itself or when

bound with homocysteine, regulates expression of a variety of genes in the *met* regulon to coordinate the activities of the two branches of the methionine biosynthetic pathway). Only *metJ* and *metH* are not known to be repressed directly by the MetJ–SAM complex (LaMonte and Hughes, 2006; Fig. 1-7b). Cells thus regulate methionine biosynthesis through a feedback mechanism that monitors levels of SAM to activate met gene expression before the concentration of methionine decreases to levels that could impair protein synthesis.

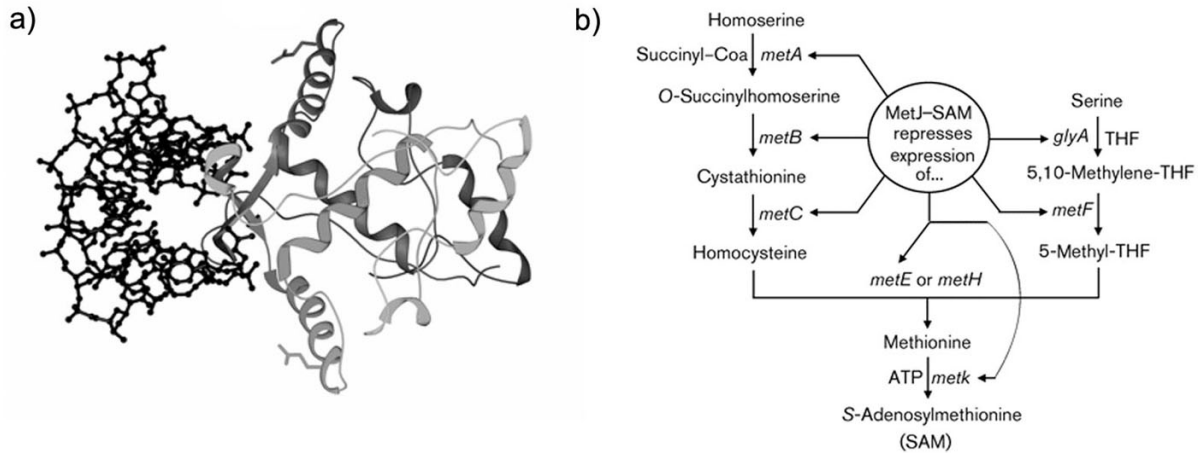


Fig 1-7 The *Eschericha coli* methionine represor MetJ; Structure of the repressor–operator complex. Cartoon representation of a single repressor dimer bound to a single metbox operator (framework model) within the higher-order complex. AdoMet is omitted for clarity (taken from Marincs et al.,2006) (**a**). MetJ role in regulation of the biosynthesis of methionine and SAM. According to the current model, the resulting SAM binds to the MetJ aporepressor to repress expression of *metA, B, C, E, F, K, R* and *glyA*; only *metJ* and *metH* are not known to be repressed directly by the MetJ–SAM complex (taken from LaMonte and Hughes, 2006) (**b**).

1.2.2 Sulphur signaling in plants – an unexplored territory

Genes involved in signaling and coordination of the response to sulphur deficiency in vascular plants remain unknown. No homolog of the *Chlamydomonas reinhardtii* SAC1 protein has been found in vascular plants (Davies et al., 1996; Leustek et al., 2000). SAC3 is similar to some kinases found in vascular plants and several of them increase in abundance in response to environmental stresses, however, their exact functions remain unknown (Anderberg and Walker-Simmons, 1992; Park et al., 1993; Yoon et al., 1997). No orthologous genes for SCON1, -2, -3 and CYS-3 of *Neurospora crassa* have been found in *Arabidopsis thaliana*. Therefore, the investigation of the regulatory system of sulphur metabolism at the level of transcription within *Arabidopsis thaliana*, as a member of higher vascular plants, is of high interest.

1.2.3 Transcription factors in *Arabidopsis*

Expression of many genes involved in sulphate uptake and assimilation is regulated at the transcriptional level. This implies transcription factors for S-regulation in plants, although little is known on this area, yet. Recently, a SLIM1/EIL3 transcription factor required for activation of sulphur assimilation and plant growth on low sulphur environment was identified by using series of sulphur

limitation response-less *Arabidopsis* mutants with a sulphur-responsive promoter-GFP reporter system (Maruyama-Nakashita et al., 2006).

Transcription factors (TF) are sequence-specific DNA-binding proteins capable of activating or/and repressing transcription of target genes by modulating the rate of initiation of mRNA synthesis by RNA polymerase II (Endt et al., 2002; Zhang, 2003, Qu and Zhu, 2006). Their domain architecture includes at least one DNA-binding domain (DBD) which mediates the binding to specific DNA sequences in the promoter region of their target genes, and a transactivation domain (TAD) that can interact with the basal transcription machinery. In many cases, additional domains mediate other interactions, such as homo- or heterodimerisation, binding of co-activators or low-molecular weight ligands (e.g. steroid hormones), interaction with other transcription factors and/or the chromatin remodelling proteins (Lewin, 2000; Broun, 2004). Such interactions with proteins of other classes can be mechanistically important for the control of transcription and they can also provide the link between transcription factor activity and signal transduction pathways, as for example in light- and disease-responses (Riechmann, 2002). Transcription factors are often expressed in a tissue-specific, developmental-stage-specific, or stimulus-specific manner (Zhang, 2003).

Many of the transcription factors might have overlapping or partially redundant functions, which can complicate their genetic analysis (Riechmann, 2002). *Arabidopsis* MYB proteins WEREWOLF and GLABROUS1 have been shown to be functionally interchangeable, and owe their particular roles in plant development to differences in their expression patterns (Lee and Schiefelbein, 2001). Transcriptional regulators in eukaryotes operate following a combinatorial logic, which is an efficient way of increasing the number and diversity of the gene regulatory activities. Often they form large multi subunit complexes, which might be necessary for their specificity and functionality (Riechmann, 2002).

The availability of *Arabidopsis* genome sequence (*Arabidopsis* Genome Initiative, 2000), allows global, genomic analysis of transcriptional regulation in plants. Initial estimates put the number of TF genes in *Arabidopsis* at 1572 TFs or approximately 6.1% of the total number of 25498 genes (Riechmann, 2002). This made the content of TF genes in *Arabidopsis* and *H. sapiens* (4.6-6.6% of total number of the genes) similar (Morgan, 2001).

More recent data, available at: http://arabidopsis.med.ohio-state.edu/AtTFDB/ and http://genetics.mgh.harvard.edu/sheenweb/AraTRs.html, enlarge this to number around 2200 genes or nearly 8% of the genome. Grouping *Arabidopsis* TF proteins according to the sequence of TF DNA-binding domains resulted in the classification of 45 families and 15 subfamilies (Fig. 1-8; according to Riechmann, 2002). In addition, there are few single-copy or “orphan” TF, such as *LEAFY* (*LFY*). Several *Arabidopsis* TF families are large and including more than 100 members. The three largest families of transcription factors in *Arabidopsis* are: AP2/EREBP (Apetala2/Ethylene Response Element Binding Protein), bHLH (basic-region Helix-Loop-Helix), and MYB-(R1)R2R3, however, each represent only ~9% of the total TF number. There are several other families with comparable numbers of genes (Riechmann et al., 2000, Riechmann, 2002). Novel TF are still being discovered and their number varies among existing databases, mainly because of differences in bioinformatic search stringency and definitions of unclassified TFs (Riechmann, 2002; Qu and Zhu, 2006).

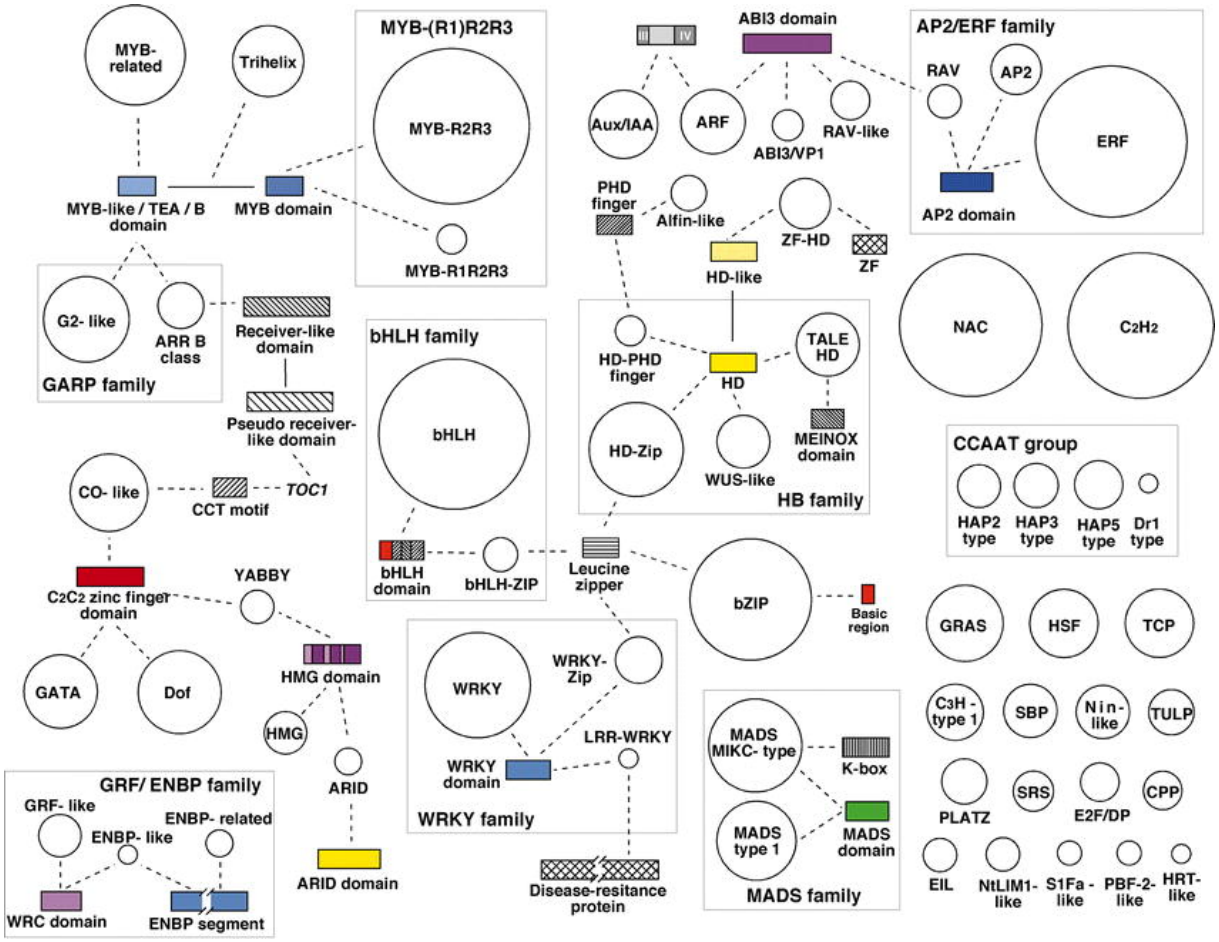


Fig 1-8 The *Arabidopsis* complement of transcription factors (taken from Riechmann, 2002). Gene families are represented by circles whose size is proportional to the number of members in the family. Domains that have been shuffled, and therefore "connect" different groups of TFs are indicated in rectangles, whose size is proportional to the length of the domain. DNA binding domains are colored; other domains (usually protein-protein interaction domains) are shown with hatched patterns. Dashed lines indicate that a given domain is a characteristic of the family to which it is connected.

The large number of transcription factors in *Arabidopsis* was interpreted in the context of the complexity of secondary metabolism in plants, which they regulate (Szathmáry et al., 2001), but it might also be related to the complex interactions between plants and the environment (both biotic and abiotic) as well as to the degree of duplications in the genome (Riechmann et al., 2000; Riechmann, 2002).

Shuffling of TF DNA-binding domains during evolution has generated novel TFs with plant-specific combinations of modules, within TF families like homeodomain, MADS or ARID. For example, combinations of the: homeobox domain with leucine zipper, PHD finger or plant specific-zinc finger domains are not found in yeast, *Drosophila,* or *C. elegans* (Riechmann et al., 2000; Riechmann, 2002). Members of kingdom-specific families represent 45% of the *Arabidopsis* complement of transcriptional regulators (Table 1.1).

Recently, a concerted large-scale cloning effort cloned 1282 ORFs encoding Arabidopsis TFs (Gong et al., 2004), and the expression profiles of over 1400 TFs were analyzed using real-time quantitative reverse transcriptase qRT-PCR technology (Czechowski et al., 2004). However, only a small fraction (around 10%) have been characterized functionally (Qu and Zhu, 2006). Most of the TF

genes were characterized through the traditional, forward genetic approach whereby genes are first defined by the mutant phenotype and then isolated. A detailed list of functionally- characterized TFs and the proposed functions for TF families are available elsewhere (Riechmann et al., 2000; Riechmann and Ratcliffe, 2000; Riechmann, 2002; Zhang, 2003; Broun, 2004). There is still very little known about the modes of TF action that is on the genes that they regulate and on the mechanisms that they use to achieve that regulation. The combinatorial nature of transcriptional regulation also adds to the complexity of this research area.

Table 1-1 Content and distribution of transcription factors in eukaryotic organisms (taken from Riechmann 2002).

Number of genes	***A. thaliana*** **25.498 [1] – 29454[2]**	***D. melanogaster*** **~14000[3]**	***C. elegans*** **~19000[4]**	***S. cerevisiae*** **~6000[5]**
Number of TFs (% of the genome)	2256[6] (5.3 – 8.6%)	642 (4.6%)	673 (3.5%)	210 (3.5%)
% of TFs from kingdom-specific families	~45	~14	~47	~32
% of TFs from families common to all three kingdoms	~53	~81	~49	~65
% of TFs from families present in two of the three kingdoms	~2	~5	~4	~3

Table legend:

[1] 2000
[2] Alonso et al., 2003
[3] Adams et al., 2000
[4] 1998
[5] Goffeau et al., 1996
[6] 2005; http://arabidopsis.med.ohio-state.edu/AtTFDB/ ; http://genetics.mgh.harvard.edu/sheenweb/AraTRs.html

1.2.4 AP2/EREBP family

In this study, two genes from AP2/EREBP TF family, *At2g28550* and *At5g60120*, are of high interest, as they were found to be involved in the regulation of sulphate metabolism.

The APETALA2 (AP2) domain (also called the AP2/ethylene-responsive element–binding factor [ERF] domain) defines a large gene family of DNA-binding proteins called AP2/ERF or AP2/EREBP (AP2/ethylene-responsive element binding protein) (Okamuro et al., 1997; Riechmann and Meyerowitz, 1998; Riechmann et al., 2000; Sakuma et al., 2002; Magnani et al., 2004). The common AP2 domain of about 60 amino acids consists of a three-stranded *β*-sheet and one *α*-helix running almost parallel to the *β*-sheet. It contacts DNA via Arg and Trp residues located in the *β*-sheet. Two conserved segments are found within each AP2 domain: the YRG element, a N-terminal stretch of 20 amino acids rich in basic and hydrophilic residues, and RAYD element, a C-terminal sequence forming amphipathic *α*-helix. It has been suggested, that YRG element could be directly involved in

DNA binding due to its basic character, while the amphipathic RAYD element could be involved in protein-protein interactions, but the possibility that it is involved in contacts with the DNA has been also considered (Okamuro et al., 1997).

The AP2 domain has been considered plant specific (Riechmann and Meyerowitz 1998). However, recent studies showed that homologues have been revealed from the cyanobacterium *Trichodesmium erythraeum*, the ciliate *Tetrahymena thermophila*, and the viruses *Enterobacteria phage* Rb49 and *Bacteriophage Felix* 01 (Magnani et al., 2004). These nonplant proteins bearing an AP2 domain are predicted to be HNH (or in some cases, HNN; histidine and asparagine) endonucleases, which through a horizontal transfer from bacteria into plants may have led to the origin of the AP2/EREBP family.

AP2/EREBP genes are divided into classes based on the number of AP2 domains that are present (Fig 1-9a; according to Kim et al., 2005). One class encodes a protein containing two AP2 domains, R1 and R2, (most of AP2-like) and includes *AP2* (Jofuku et al. 1994), *AINTEGUMENTA* (*ANT*) (Elliott et al. 1996; Klucher et al. 1996), and *Glossy15* (*GL15*) (Moose and Sisco 1996). A second class encodes a protein with only one AP2 domain, R1 or R2, (most of EREBP-like) and includes *ERFs* (Ohme-Takagi and Shinshi 1995), *TINY* (Wilson et al. 1996), *AtEBP* (Buttner and Singh 1997), and *ABI4* (Finkelstein et al. 1998). A third class of AP2/ERF genes, RAV1 and RAV2 (Kagaya, Ohmiya, and Hattori 1999), encodes proteins that have two different DNA-binding domains, AP2 and B3 (Giraudat et al. 1992).

The subdivision of the multigene AP2/EREBP family was based also on the different exon/intron structure of AP2-like and EREBP-like genes. AP2-like genes contain multiple exons and, in particular, both AP2 domains of each protein are encoded by several exons. In contrast, the sequences coding for the AP2 domain in EREBP-like genes are not interrupted by introns. This has been shown for *TINY* and *RAP2.10*, which are intronless genes (Wilson et al., 1996; Riechmann and Meyerowitz, 1998).

Following the complete sequencing of the Arabidopsis genome, Riechmann et al. (2000) searched for AP2/EREBP in the *Arabidopsis* genome and found 144 AP2/EREBP genes. Sakuma et al. (2002) classified AP2/EREBP genes in Arabidopsis as members of five classes based on similarities in their DNA-binding domains: AP2 subfamily (14 genes), RAV subfamily (6 genes), DREB subfamily (55 genes), ERF subfamily (65 genes), and others (the fifth group; 4 genes). Recently, a total of 161 AP2 domains were extracted from 147 hypothetical proteins obtained from a database search and multiple sequence alignment was performed (Fig. 1-9b; according to Feng et all., 2005)

The AP2-like genes whose functions have been determined by mutant analyses (e.g., Arabidopsis *AP2, ANT*, and maize *GL15*) act as key regulators in developmental processes, whereas the EREBP-like genes (e.g., tobacco ethylene-responsive element–binding protein [EREBP]-2) appear to be involved in responses to biotic and environmental stress (Riechmann and Meyerowitz 1998).

Arabidopsis *AP2* is the most well-studied gene in AP2/EREBP family. *AP2* encodes a putative transcription factor (Jofuku et al. 1994; Riechmann and Meyerowitz 1998) and plays a central role in the establishment of the floral meristem (Irish and Sussex 1990; Huala and Sussex 1992; Bowman et al. 1993; Schultz and Haughn 1993; Shannon and Meekswagner 1993), the specification of floral

organ identity (Komaki et al. 1988; Bowman, Smyth, and Meyerowitz 1989; Kunst et al. 1989), and the regulation of floral homeotic gene expression (Bowman, Drews, and Meyerowitz 1991; Drews, Bowman, and Meyerowitz 1991; Jack, Brockman, and Meyerowitz 1992; Mandel et al. 1992) in *Arabidopsis*. In addition to *AP2*, other genes encoding the AP2 domain have been well studied in *Arabidopsis*. These include *ANT* (AP2-like), a gene that regulates ovule development and floral organ growth (Elliott et al. 1996; Klucher et al. 1996), and *CBF1* (ERF-like), a gene that binds to the C-repeat/Dehydration Response Element, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit (Stockinger et al. 1997).

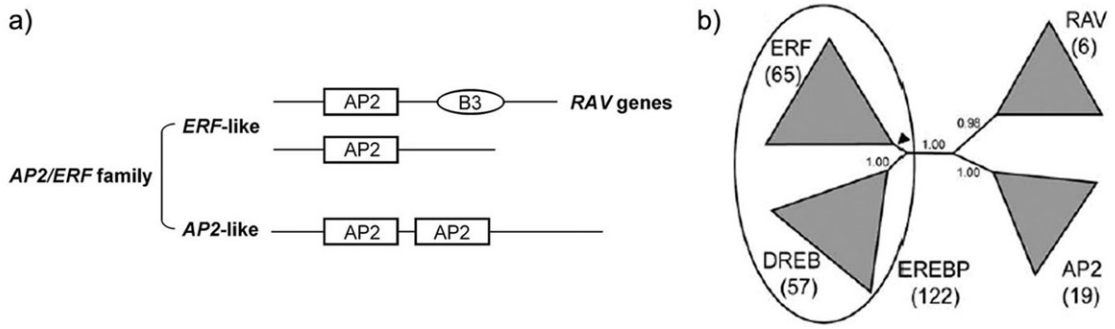


Fig 1-9 Structure of AP2/EREBP genes. ERF-like genes contain one AP2 domain, AP2-like genes contain two AP2 domains and RAV-like genes contain one AP2 and one B3 domain (taken from Kim et al., 2005) (**a**). Phylogenetic analyses of AP2/EREBP TF genes from the Arabidopsis genome. Major clades depict the relationships among different subfamilies. The number of genes in each subfamily or subgroup is shown in brackets (taken from Feng et al., 2005) (**b**).

DNA binding specificity has been shown for members of the ERF, DREB, AP2, and RAV subfamilies. Several ERF proteins bind the GCC box (AGCCGCC) where G2, G5, and C7 are essential for binding (Ohme-Takagi and Shinshi, 1995; Buttner and Singh, 1997; Zhou et al., 1997; Hao et al., 1998; Fujimoto et al., 2000; Hao et al., 2002). The dehydration response element ([DRE], TACCGACAT) is recognized by proteins of the DREB subfamily (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). The sequence CCGAC inside the DRE element is the minimal sequence motif for binding, and C4, G5, and C7 are essential for specific interaction (Hao et al., 2002; Sakuma et al., 2002). DREB factors are known to also bind the C-repeat and the low-temperature-responsive element, which share the CCGAC motif with the DRE element (Baker et al., 1994; Jiang et al., 1996; Thomashow, 1999). The Arabidopsis RAV1 transcription factor can bind a bipartite recognition sequence with the B3 and the AP2 domain recognizing the sequences CACCTG and CAACA, respectively (Kagaya et al., 1999). The only member of the AP2 subfamily with a characterized binding sequence is the Arabidopsis AINTEGUMENTA (ANT) protein. The two AP2 domains in ANT selectively bind the consensus sequence gCAC(A/G)N(A/T)TcCC(a/g)ANG(c/t) (Nole-Wilson and Krizek, 2000; Krizek, 2003).

Two genes from AP2/EREBP, *At2g28550* and *At5g60120*, were investigated in this study. Both these genes belong to the AP2-like subfamily, however, sequence analysis revealed the presence of two AP2 domains in the *At2g28550* gene, while the *At5g60120* gene possess only one AP2 domain (R1) (Fig. 1-10; according to Feng et al., 2005). Interestingly, the AP2 domain of *At5g60120* gene is the closest neighbour of R1 domain of the *At2g28550* gene, since they share high

sequence similarity on the protein maximum likelihood tree. Table 1-2 summarizes research done on both these AP2 genes to date.

Table1-2 Research about *At2g28550* and *At5g60120* genes published to date.

AGI code	Gene name	Description	References	Code [a]
At2g28550	RAP2.7 /TOE1	Plant defense response and floral organogenesis.	Aukerman and Sakai (2003), *Plant cell* 15: 2730-2741, Krizek et al. (2000), Schneitz et al., 1998, Okamuro et al. (1997), *Proc. Natl. Acad. Sci.* 94: 7076-7081, Klucher et al. (1996), *Plant Cell* 8: 137-156; Feng et al. (2005) *Plant Mol Biol* 59: 853–868	# 63
At5g60120	TOE2	Flower development	Aukerman and Sakai (2003) *Plant cell* 15: 2730-2741; Feng et al. (2005) *Plant Mol Biol* 59: 853–868	# 70

Table legend:
[a] code used in this study

Feng et al. (2005) examined tissue expression patterns and environmental or hormonal regulations of AP2 TF gene family by using both oligo microarray and custom cDNA macroarray. Comparing to rosette leaves, the transcript levels of the *At2g28550* gene was found to decrease more than 16-fold in reproductive tissues, such as sepal, petal, stamen, pistil and silique, 2-fold in seed, hypocotyl, stem and cauline leaf, while in cotyledon and root these differences were smaller varying between ±2-fold. For the gene *At5g60120* the highest expression was found in roots (2 to 8-fold comparing to rosette leaves), the lowest (2 to 16-fold) for C-leaf, stem, sepal, petal and stamen. Hormonal and environmental treatment revealed 2,55-fold induction of the gene *At2g28550* by cold and 2,17-fold repression by UV treatment, while other treatments did not change this gene expression more than 2-fold. Also none of the treatments implemented by authors did not result in activation or repression of the gene *At5g60120*. Hormonal or environmental responsiveness of both AP2 genes reported by Feng et al. (2005) mostly match those reported in the *Genevestigator* database (https://www.genevestigator.ethz.ch/), which comprises thousands of Arabidopsis arrays processed to date (Zimmermann et al., 2004). Searching the public available arrays (ATH1 full genome arrays and Nottingham Arabidopsis Stock Centre Transcriptomics Service arrays) revealed the highest induction of the *At2g28550* gene by cold (3,87-fold), but also by all kinds of light treatment, such as blue and far red light (both 3,12-fold), white (2,98-fold), red (2,7-fold), UV-A (2,31-fold) and UV-AB (2,32-fold). Among nutrients stresses, potassium deprivation repressed the *At2g28550* gene expression 2,2 times, nitrogen depravation induced it slightly (1,38-fold), while sulphate depravation had no effect (0,84-fold). Ethylene treatment resulted in decreased transcript level of the *At2g28550* gene 2,4-fold, comparing to untreated plants. Results obtained for the gene *At5g60120* revealed, that any treatment did not induced or repressed its expression greater than 2-fold.

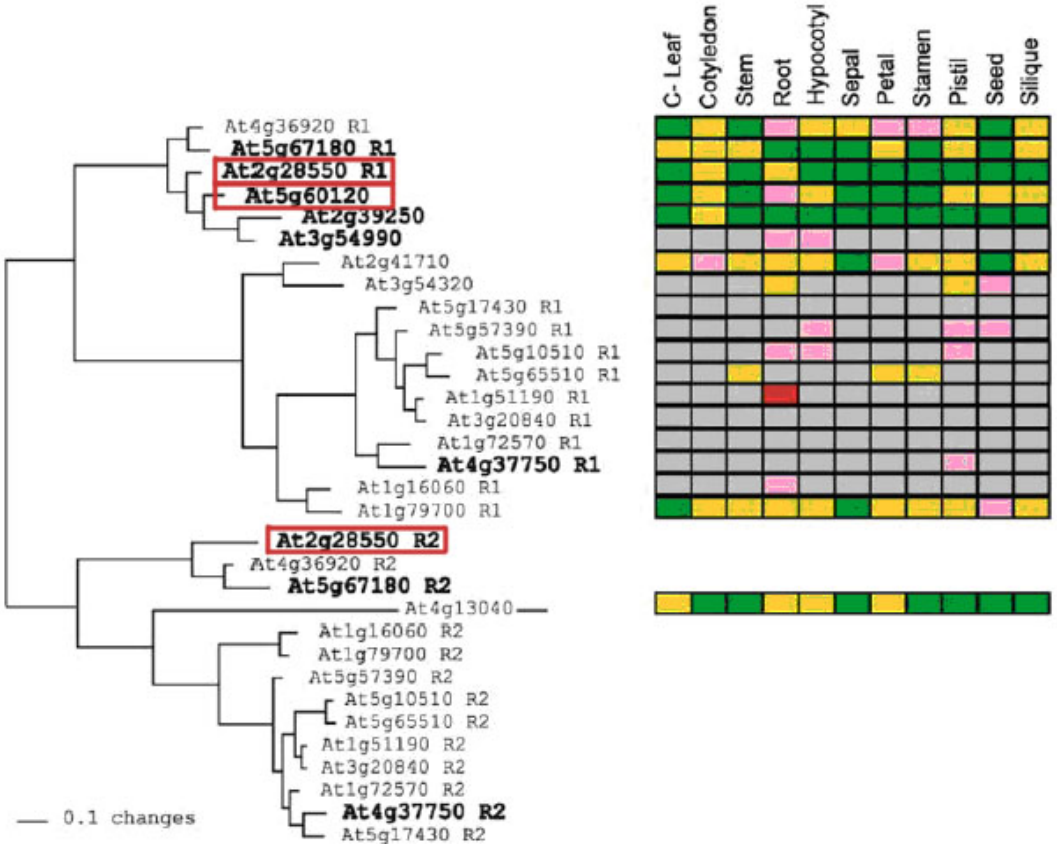


Fig 1-10 Construction of protein maximum likelihood tree for the AP2-like subfamily and expressional profiling obtained from oligo microarray (taken from Feng et al., 2005). The Ap2-like subfamily comprises of 19 genes and 32 AP2 domains (13 of these proteins contain two AP2 domains, and 6 of them contain only one AP2 domain). *At4g13040* was the only gene that possessed a single R2 domain. Unrooted tree for 32 AP2 domains is shown on the left and results obtained from oligo microarray analysis of 19 TFs in various Arabidopsis tissue or organ types are shown on the right. Ratios obtained by dividing the hybridizing intensity from a particular tissue or organ type with that of rosette leaves for a particular TF was subjected to logarithmic transformation and used as a graphic unit. Red bars, genes increased more than 8-fold; pink bars, genes increased 2- to 8-fold; yellow bars, genes changed between±2-fold; green bars, genes decreased more than 2-fold. Genes that either failed the T-test (at the 0.05 significance level) or produced total signal intensities below threshold are shown in gray and were considered as non-expressers.

Searching the TAIR database (http://www.arabidopsis.org/) indicated that the *At2g28550* gene is known to be expressed in two splice variants, which are known as a RAP2.7 and TOE1 proteins. *At5g60120* is expressed as a TOE2 protein. TOE1 and TOE2 act as floral repressors. Overexpression of *TOE1* causes late flowering phenotype (Aukerman and Sakai, 2003). Homozygous *toe1-2* T-DNA insertion mutants were slightly early flowering relative to the wild type, whereas *toe2-1* plants were not significantly early flowering. However, the *toe1-2 toe2-1* double mutant was earlier flowering than either individual mutant, suggesting that the genes have overlapping function (Aukerman and Sakai, 2003). Both *TOE1* and *TOE2* genes were found to be regulated by *miRNA172* (Park et al., 2002; Chen, 2003; Jones-Rhoades and Bartel, 2004) in a translational mechanism (Aukerman and Sakai, 2003). *miR172* appeared to down-regulate their AP2 gene targets, as it caused early flowering and defects in floral organ identity when overexpressed. The early flowering phenotype of *toe1-2 toe2-1* is consistent with a postulated model, in which miR172 causes early flowering by down-regulating *TOE1* and *TOE2* floral repressors.

1.2.5 WRKY family

WRKY proteins are recently identified transcriptional regulators comprising a large gene family with 72 representatives in *Arabidopsis* (Eulgem et al., 2000; Riechmann, 2002). The first WRKY cDNAs were cloned from sweet potato (*Ipomoea batatas*; SPF1), wild oat (*Avena fatua*; ABF1,2), parsley (*Petroselinum crispum*; *Pc*WRKY1,2,3) and *Arabidopsis* (ZAP1) (Eulgem et al., 2000). A single copy of the WRKY gene encoding two WRKY domains was identified from *Giardia lamblia*, a primitive eukaryote, *Dictyostelium discoideum*, a slime mold closely related to the lineage of animals and fungi, and the green alga *Chlamydomonas reinhardtii*, an early branching of plants. This ancestral WRKY gene seems to have duplicated many times during the evolution of plants, resulting in a large family in evolutionarily advanced flowering plants (Zhang and Wang, 2005). To date, WRKY genes have not been cloned from species other than plants. The absence of WRKY homologues in the genomes of animals (*Caenorhabditis elegans* and *Drosophila melanogaster*) and yeast (*Saccharomyces cerevisiae*) leads to the suggestion that WRKY transcription regulators are restricted to the plant kingdom (Riechmann et al., 2000; Eulgem et al., 2000).

The WRKY family proteins contain one or two highly conserved WRKY domains characterized by the hallmark heptapeptide WRKYGQK and a zinc-finger structure distinct from other known zinc-finger motifs (Eulgem et al., 2000). To regulate gene expression, the WRKY domain binds specifically *cis*-acting DNA sequence motif (T)(T)TGAC(C/T), which is known as the W box (de Pater et al., 1996; Rushton et al., 1995; 2002). In addition to the W box, a recent study indicates that the WRKY domain can also bind to SURE, a sugar responsive *cis* element, as a transcription activator (Sun et al., 2003).

Based on the number of WRKY domains and the pattern of the zinc-finger motif, Eulgem et al. (2000) classified members of the WRKY superfamily from the *Arabidopsis* genome into three groups. Members of Group 1 typically contain two WRKY domains, while most proteins with one WRKY domain belong to Group 2. Group 3 proteins also have a single WRKY domain, but the pattern of the zinc-finger motif is unique. Eulgem et al. (2000) further divided Group 2 into five subgroups, according to the phylogenetic analysis of the WRKY domains.

In plants, many WRKY proteins are involved in the defense against attack from pathogenic bacteria (Du and Chen, 2000; Deslandes et al., 2002; Chen et al., 2002; Chen and Chen, 2002; Dong et al., 2003), fungi (Chen et al., 2002), viruses (Wang et al., 1998; Yang et al., 1999; Chen et al., 2002), and oomycetes (Chen et al., 2002; Kalde et al., 2003). Further, WRKY genes are implicated in responses to the abiotic stresses of wounding, being induced by signaling substances such as salicylic acid (Hara et al., 2000; Cheong et al., 2002), the combination of drought and heat (Rizhsky et al., 2002), and cold (Huang and Duman, 2002). It is also evident that some members of the family may play important regulatory roles in morphogenesis of trichomes (Johnson et al., 2002) and embryos (Alexandrova and Conger, 2002), senescence (Chen et al., 2002; Robatzek and Somssich, 2001; 2002), dormancy (Pnueli et al., 2002), plant growth (Chen and Chen, 2002), and metabolic pathways (Rushton et al., 1995; Willmott et al., 1998; Johnson et al., 2002; Sun et al., 2003).

In this study, a member of the WRKY TF family, the *At5g41570* gene was found to be sulphur-sensitive. Searching the TAIR database (http://www.arabidopsis.org/) indicated that the *At5g41570*

gene is known to be expressed in two splice variants, a WRKY24 protein and a protein identical to the WRKY24. Phylogenic analysis done on amino acids sequences of 58 members of the *Arabidopsis* WRKY TF revealed that the WRKY24 protein possesses one WRKY domain and belongs to the 2C WRKY sub-family (Eulgem et al., 2000). Although several T-DNA insertional mutants spanning exon region of the *At5g41570* gene are available, none of them has been reported to give visible phenotype aberrance, according to TAIR database (www.arabidopsis.org). No EST for the *At5g41570* gene is spotted on the ATH1 Affymetrix chip, thus, no expressional data can be obtained for these gene by searching the public available arrays.

1.2.6 MYB family

The MYB family of proteins is a group of functionally diverse proteins found in both plants and animals (Rosinski and Atchley, 1998). The first MYB gene identified was the v-MYB gene of avian myeloblastosis virus (AMV) (Klempnauer et al., 1982). Three v-MYB-related genes were subsequently found in many vertebrates (Weston, 1998) and homologous genes were also identified in insects, fungi, and slime molds (Lipsick, 1996). The first plant MYB gene, *C1*, was isolated from *Zea mays*, and it was shown to be involved in anthocyanin biosynthesis (Paz-Ares et al., 1987). The fact that MYB genes exist widely in eukaryotes suggests that these genes may be very ancient during the evolutionary course. Interestingly, the numbers of MYB genes in plants are remarkably higher than those in fungi or animals (Riechmann et al., 2000).

A MYB domain is a region of about 52 amino acids, usually composed of one to three imperfect repeats that adopt a helix-turn-helix conformation to intercalate in the major groove of the DNA. For example, the MYB domain of the well characterized mammalian transcription factor c-MYB is composed of the three repeats, R1, R2, and R3 (Paz-Ares et al., 1987). Typically, three regularly spaced tryptophan residues are present in each MYB repeat, participating in a hydrophobic cluster that is presumably involved in the specific recognition of DNA (Ogata et al., 1995). In most organisms, the MYB domain binds to a specific DNA sequence C/TAACG/TG. Plant MYB proteins were classified into three major groups: R2R3-MYB, with two adjacent repeats; R1R2R3-MYB, with three adjacent repeats; and a heterogeneous group collectively referred to as the MYB-related proteins, which usually but not always contain a single MYB repeat (Rosinski and Atchley, 1998; Jin and Martin, 1999; Stracke et al., 2001).

The MYB superfamily has the largest number of members of any *Arabidopsis* gene family (Riechmann and Ratcliffe, 2000). Recently, 198 genes of the MYB superfamily were identified, based on analysis of the complete *Arabidopsis* genome sequence, among them, 126 are R2R3-MYB, 5 are R1R2R3-MYB, 64 are MYB-related, and 3 atypical MYB genes (Yanhui et al., 2006; see also Fig 1-8).

In the past decade, the R2R3-MYB genes have been extensively studied. They were reported to be involved in many physiological and biochemical processes, such as the regulation of secondary metabolism (Paz-Ares et al., 1987; Bender and Fink, 1998; Hoeren et al., 1998; Borevitz et al., 2000; Jin et al., 2000; Nesi et al., 2001; Baudry et al., 2004), the control of cell morphogenesis (Oppenheimer et al., 1991, Noda et al., 1994; Glover et al., 1998; Lee and Schiefelbein, 1999, 2001;

Higginson et al., 2003), the regulation of meristem formation and floral and seed development (Kirik et al., 1998; Penfield et al., 2001; Shin et al., 2002; Steiner-Lange, 2003), and the control of cell cycle (Ito et al., 2001; Araki et al., 2004). Some were also involved in various defense and stress responses (Urao et al., 1993; Hemm et al., 2001; Stockinger et al., 2001; Vailleau et al., 2002; Abe et al., 2003; Denekamp and Smeekens, 2003; Nagaoka and Takano, 2003) and in light and hormone signaling pathways (Wang et al., 1997; Ballesteros et al., 2001; Gocal et al., 2001; Seo et al., 2003, Newman et al., 2004).

A well characterised R2R3-MYB transcription factor, PAP1 (MYB75; *At1g56650*), was found to be involved in anthocyanin biosynthesis and metabolism, response to salt stress, removal of superoxide radicals, sucrose mediated signaling, and response to jasmonic acid stimulus (Teng et al., 2005; Sharma and Dixon et al., 2005; Tohge et al., 2005a; 2005b; Baudry et al., 2006; Pourtau et al., 2006). In this study *PAP1* gene was shown to react strongly in its expression to SO_4^{2+} deficiency but also to other stress conditions applied, thus, it was considered as a general stress-responsive factor.

Another R2R3-MYB gene, the *At1g34670* gene was also found in this study to be sulphur-sensitive. According to TAIR database (http://www.arabidopsis.org/), it is known to be expressed as a MYB93 protein. The 'reverse Northern' study of organ-specific expression pattern of *Arabidopsis* MYB genes revealed that the *At1g34670* gene is expressed weakly and exclusively in roots, while among various treatments applied, the *At1g34670* gene reacted weakly to auxin and abscisic acid stimulus and to low nitrate and high sucrose treatments (Kranz et al., 1998). The hormone treatment results concerning *MYB93* gene expression and its organ-specificity were confirmed by Yanhui et al. (2006), who additionally found the *MYB93* to be SA-sensitive. Hormonal or environmental responsiveness of *MYB93* gene reported by Kranz et al. (1998) and Yanhui et al. (2006) fully match those reported in the *Genevestigator* database (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004). Searching the public available *Arabidopsis* arrays (ATH1 full genome arrays and Nottingham Arabidopsis Stock Centre Transcriptomics Service arrays) revealed the weak induction of *MYB93* by ABA (1,8-fold), IAA (1,61-fold) and high sucrose (2,26-fold). The highest induction of *MYB93* gene was revealed by nitrate deprivation experiments and reached 7,75-fold, when compared to control conditions (signal levels near background, thus, ratio might be artificial). Array experiments provided by *Genevestigator* did not reveal any response of *MYB93* to sulphate starvation. Osmotic and salt stress resulted in slightly elevated transcript levels of the *At1g34670* gene (1,41 and 1,31-fold, respectively). High induction of *MYB93* gene was obtained after treatment with *A. tumefaciens* (4,55-fold) and hydrogen peroxide (2,42-fold). Almost 1,6-fold decrease in *MYB93* expression was revealed after cold treatment and during senescence. To date, the *At1g34670* gene was not reported to be studied by reverse genetic approaches.

1.2.7 *cis*-acting components involved in transcriptional control of sulphate status in *Arabidopsis*

Given the apparent importance of transcriptional regulation, a priority is the identification of components of the signal transduction pathway. Potential sulphur-responsive elements (SUREs) in

promoter regions have been described. The 16-bp sulphur responsive element (SURE) from –2777 to –2762 of *SULTR1;1* promoter was sufficient and necessary for the –S-responsive expression (Maruyama-Nakashita et al., 2005). The SURE sequence contained an auxin response factor (ARF) binding sequence (GAGACA). However, SURE was not responsive to naphthalene acetic acid, indicating its specific function in the sulphur response. The base substitution analysis indicated the significance of a 5-bp sequence (GAGAC) within the conserved ARF binding site as a core element for the –S response. Microarray analysis of early –S response in *Arabidopsis* roots indicated the presence of SURE core sequences in the promoter regions of many –S-inducible genes, such as *SULTR2;1* (*At5g10180*), NADPH oxidoreductase (*At1g75290*), putative β-thioglucosidase (*At2g44460*), *SULTR4;2* (*At3g12520*) and *APR3* (*At4g219960*). It is suggested that SURE core sequences may commonly regulate the expression of a gene set required for adaptation to the –S environment.

1.2.8 Regulation of nitrate and phosphate metabolism by transcription factors

In the last decade, plant responses to nitrate and phosphate deprivation were studied extensively (Wang et al., 2000; 2003; 2004; Scheible et al., 2004; Wu et al., 2003; Hammond et al., 2003; 2004; Muller et al., 2007; Morcuende et al., 2007). Several array experiments revealed TF genes that respond to nitrogen or phosphate deprivation, but few of them has been so far characterised functionally.

Microarray analysis comparing *Arabidopsis* seedlings grown on high (10 mM) or low (0.5 mM) nitrate, supplemented with 5 mM Gln, showed strong response to nitrate for two transcription factors: *bZIP-210* (bZIP family member) and *ATL2-237* (LIM family member) (Tranbarger et al., 2003). The same work showed that their expression was preferentially observed in roots and correlates to the root response to nitrate availability.

Genetic screening of the homozygous T-DNA insertion lines for 23 members of *Arabidopsis* GATA TF family allowed to identify one line with an altered phenotype observed in defined growth conditions (Bi et al., 2005). In this line, the T-DNA insertion in the second exon of *At5g56860* prevents the expression of the GATA domain. In wild-type plants, the expression of *At5g56860* is shoot-specific, occurs at an early stage of development and is inducible by nitrate. Loss of expression of *At5g56860* in the loss-of-function mutant plants resulted in reduced chlorophyll levels. A transcript profiling experiment revealed that a considerable proportion of genes down-regulated in the loss-of-function mutants are involved in carbon metabolism and *At5g56860* is thus designated GNC (GATA, Nitrate-inducible, Carbon metabolism-involved). gnc mutants are more sensitive to exogenous glucose, and two hexose transporter genes, with a possible connection to glucose signaling, are significantly down-regulated, while GNC over-expressing transgenic plants up-regulate their expression and are less sensitive to exogenous glucose. These observations suggest a function for GNC in regulating carbon and nitrogen metabolism.

Lea et al. (2006) examined nitrogen effects on expression of the various regulators in the flavonoid pathway. Four out of eight regulators involved in the flavonoid pathway showed an enhanced expression from 2 to 1,000 times in response to nitrogen deficiency. *PAP1* and *PAP2* were up-

regulated in response to nitrogen deficiency in wild type as well as *pap1D* plants (a T-DNA activation-tagged line overexpressing the *PAP1/MYB75* gene). At least three different bHLH domain transcription factors promote anthocyanin synthesis, and transcripts for one of these, i.e. *GL3* were found to be sixfold enhanced by nitrogen deficiency. The MYB12 transcription factor, known to regulate flavonol synthesis, was slightly induced by nitrogen deficiency. Together with MYB factors, especially PAP2, GL3 appears to be the BHLH partner for anthocyanin accumulation in response to nitrogen deficiency.

In *Chlamydomonas reinhardtii*, a nuclear-localised MYB-protein, Psr1, is known to be induced by phosphate deficiency and is essential for the activation of phosphate assimilatory genes (Wykoff et al. 1999). Using the *Chlamydomonas Psr1* sequence as a query, two *Arabidopsis* homologs, *At1g79430* and *At3g04030*, were identified (Todd et al., 2004). The predicted amino acid sequences of both coding regions contain MYB-like and α-helical domains. Both transcripts, *AtPhr2* and *AtNsr1* in *Arabidopsis*, are responsive to P and N nutrient status, respectively. T-DNA disruption of *AtNsr1* resulted in altered expression of at least one nitrate transporter (*AtNRT2.5*). Further, the *pho2-1/pho2-1* mutant, reported to be a phosphate accumulator, showed no increase in *AtPhr2* mRNA in response to –P and a 70% reduction in the response of *AtNsr1* mRNA to –N.

A clear role has been established for *PHR1* in P signalling. This MYB-like transcription factor also has homology to *PSR1* from *Chlamydomonas reinhardtii* (Wykoff et al. 1999), and regulates the expression of target genes like acid phosphatase (*AtACP5*), *AtIPS1*, *PHT1.1* and *RNS1* (Martin et al. 2000; Rubio et al. 2001), and other Pi starvation-induced genes including microRNA399 genes (Bari et al. 2006) by binding to an imperfect palindromic 8-bp sequence (GNATATNC) in their promoter (Rubio et al. 2001; Franco-Zorilla et al. 2004; Hammond, Broadley and White 2004).

Another transcriptor factor potentially related to phosphorus metabolism was found in screening for mutants altered in Pi starvation regulation (Rubio et al., 2001). Screening of EMS-mutagenized M2 population of an *Arabidopsis thaliana* transgenic line harboring a reporter gene specifically responsive to Pi starvation (*AtIPS1::GUS*) revealed that one of the mutants, *phr1* (*phosphate starvation response 1*), displayed reduced response of *AtIPS1::GUS* to Pi starvation, and also had a broad range of Pi starvation responses impaired, including the responsiveness of various other Pi starvation-induced genes and metabolic responses, such as the increase in anthocyanin accumulation. *PHR1* shown be related to the *PHOSPHORUS STARVATION RESPONSE 1* (*PSR1*) gene from *Chlamydomonas reinhardtii*. *PHR1* is expressed in Pi sufficient conditions and, in contrast to *PSR1*, is only weakly responsive to Pi starvation. PHR1, PSR1, and other members of the protein family share a MYB domain and a predicted coiled–coil (CC) domain, defining a subtype within the MYB superfamily, the MYB–CC family. PHR1-binding sequences are present in the promoter of Pi starvation-responsive structural genes, indicating that this protein acts downstream in the Pi starvation signaling pathway.

The Arabidopsis ANR1 gene, encoding a member of the MADS-box family of transcription factors, has been identified as a component of a signaling pathway that regulates lateral root growth in response to changes in the external NO_3 supply (Zhang and Forde, 1998). Gan et al. (2005) used quantitative real-time PCR to investigate the responsiveness of *ANR1* and 11 other root-expressed MADS-box genes to fluctuations in the supply of N, P and S. *ANR1* expression in roots of

hydroponically grown *Arabidopsis* plants was specifically regulated by changes in the N supply, being induced by N deprivation and rapidly repressed by N re-supply. Seven of the other MADS-box genes responded to N in a manner similar to *ANR1*, but less strongly, while four were unaffected. Only *SOC1* was additionally found to respond to changes in the P and S supply, suggesting a possible role in a general response to nutrient stress.

Genome-wide studies of *Arabidopsis* seedlings responding to nitrogen revealed 93 TFs which showed marked (>3-fold) changes in transcript abundance (Scheible et al., 2004). Among them the most abundantly was represented the MYB family, having 11 TFs considerably changed (including *PAP1* and *PAP2*), 6 TFs belonged to the G2-like (MYB-like) GARP family, and 7 to NIN-like family. Interestingly, NIN-like TFs contain a domain called RWP-RK after a conserved motif at the C-terminus of the domain, which was found in plant proteins involved in N-controlled development of symbiotic root nodules (Schauser at al., 1999). However, these studies failed to confirm the response of the *ANR1* gene (Zhang and Forde, 1998) and two genes reported by Tranbarger et al. (2003) to the NO_3^- availability.

Genome-wide studies of *Arabidopsis* seedlings responding to phosphorus revealed that the number of P-responsive TR genes and the magnitude of their response to altered P are much smaller than those observed by Scheible *et al*. (2004) in response to N (Morcuende et al., 2007).There were only two transcription regulator genes revealed by ATH1 chip hybridisation that displayed over 10-fold changes in transcript abundance between +P and -P conditions. However, authors proposed a set of 20 TF, for which reproducibly P responsive was found by qRT-PCR profiling.

1.3 Aims of the thesis

The aim of this thesis was to identify and characterise transcription factors that control sulphate metabolism in *Arabidopsis thaliana*. To achieve that goal we postulated that factors regulating *Arabidopsis* responses to inorganic sulphate deficiency change their transcriptional levels under sulphur-limited conditions.

By comparing TF transcript profiles from plants grown on different sulphate regimes, we aimed at identifying TF genes that may specifically induce or repress changes in expression of genes that allow plants to adapt to changes in sulphate availability. Candidate genes obtained from this screening were tested by reverse genetics approaches. We try to link their function to plant S-metabolism (section 3A).

Several published array experiments revealed TF genes that respond to sulphate deprivation, but none of these have been so far been characterized functionally. Nikiforova et al. (2003) identified two AP2-related TF as reacting to the long term sulphur-starvation. By comparing metabolite and transcript profiles from transgenic and wild type plants we aimed at confirming the role of selected AP2 TF candidate genes in plant long-term adaptation to sulphur unavailability (section 3B).

2 MATERIALS AND METHODS

2.1 Commonly used equipment, kits and consumables

2.1.1 Equipment

Applied Biosystems, Foster City, USA; 2 X ABI Prism 7900HT and 7300 real-time PCR systems,
Agilient Technologies, Waldbronn, Germany; Agilent 2100 BioAnalyser and RNA 6000 Nano Chips,
Beckman Instruments Inc., Fullerton, USA; Avanti J30I centrifuge,
Biometra, Göttingen, Germany; UNO II PCR and T Gradient machines,
Bio-Rad, Richmond, USA: gel chambers, Gel Doc, PCR machine
Dionex, Germering, Germany; ICS-2000 Ion Chromatography System, ASI-100 Automated Sample Injector, Degasys DG1210, P580 Pump, RF2000 Fluorescence Detector, Chromeleon® software,
Eppendorf, Hamburg, Germany; Microcentrifuges: 5417, 5417C, 5417R, Megefuge 5810R, BioPhotometer, SpeedVac
Knauer, Berlin, Germany; Eurosphere C_{18} column, Hypersil ODS C_{18} column
Perkin Elmer, Frankfurt/Main, Germany; Evolution P3 liquid handling system
Retsch, Haan, Germany**;** MM200 homogeniser,
NanoDrop, Wilmington, USA**;** NanoDrop ND-1000 spectrophotometer,
Satorius, Goettingen, Germany; balances

2.1.2 Consumables

AB Gene, Hamburg, Germany **;** 96 well PCR plates, adhesive PCR seals,
Alltech Grom GmbH, Rottenburg-Hailfingen**,** Germany; ortho-phthaldialdehyde (OPA), borat buffer,
Applied Biosystems, Foster City, USA; SYBR Green PCR mix, 384 well plates and adhesive covers,
Bayer CropScience AG, Monheim, Germany; Basta herbicide
Calbiochem, Darmstadt, Germany; THIOLYTE® (Monobromobimane Reagent),
Eurogentec, Seraing, Belgium; 96 well PCR plates optical grade with caps, Smart™ DNA ladder, oligonucleotides,
Fluca, Buchs, Switzerland; formamide, isopropanol, β-mercapto ethanol,
Invitrogen, Karlsruhe, Germany; RNAsin inhibitor
MWG, Ebersberg, Germany; oligonucleotides,
Merck, Darmstadt, Germany; Methanol Lichrosolv; Perchloric acid; other chemicals,
Promega, Mannheim, Germany; Oligo(dT)15 primer,

Sigma Aldrich, Taufkirchen, Germany; RNase free DNase I, Ethidiumbromide, Diethylpyrocarbonate (DEPC), amino acids standards, 1-aminocyclopropane-1-carboxylic acid (ACC), other chemicals,

Sigma, Munich, Germany; Dansyl chloride Dansychloride 95% TLC; Diaminohexan; Proline

Stratagene, Heidelberg, Germany; *Pfu* DNA polymerase,

Roche Applied Science, Hague Road, USA; antibiotics, DNAse I RNAse-free

Roth, Karlsruhe, Germany; other chemicals,

2.1.3 Kits

Affymetrix, Santa Clara, USA; ATH1 chips

Invitrogen, Karlsruhe, Germany; TRIzol™ reagent, Superscript™III reverse transcriptase, *Taq* polymerase,

Qiagen, Hilden, Germany; Oligotex mRNA Mini Kit, RNeasy Mini Kit

2.2 Media, growing conditions and plant lines

2.2.1 Plant material

2.2.1.1 Wild type

In all experiments the *Arabidopsis thaliana* (L.) ecotype Col-O was used. All transformed lines were produced in a background of *Arabidopsis thaliana* (L.) ecotype Col-O.

2.2.1.2. 35S overexpressors lines

Seeds of *Arabidopsis thaliana* plant lines overexpressing two different AP2 transcription factor genes were provided by co-workers (Isabell Witt and Maria Ines Zanor) routinely transforming *Arabidopsis thaliana* plants with the 35S promoter constructs containing TFs from different families.

Table 2-1 *Arabidopsis thaliana* plant overexpressing lines. The AGI code describes the gene the plants were transformed with, the gene descriptions are taken from The Arabidopsis Information Resource (TAIR).

AGI code	Gene name	Clone/ line number	Gene description
At2g28550	*TOE1*	# 63	AP2 domain-containing transcription factor (RAP2.7)
At5g60120	*TOE2*	# 70	AP2 domain-containing transcription factor, putative

2.2.1.3 T-DNA knockout lines

Seeds of T-DNA knockout lines were ordered from T-DNA insertion bank Salk Institute Genomic Analysis Laboratory (SIGnAL; http://signal.salk.edu/), which provide T-DNA knock out mutant lines in exactly determined positions of the genome. Used plant lines are listed in table 2-2. They all represent transcription factors belonging to different transcription factor families (e.g. MYB, AP2, NAC). In some cases the correct function is not known but the proteins show, at least a putative, specific DNA-binding region.

Table 2-2 List of *Arabidopsis thaliana* knockout lines: AGI code describes localization in the genome and chromosome, line names are given by SALK and descriptions are from The Arabidopsis Information Resource (TAIR)

AGI code	line number	SALK line	insertion region	gene description
At2g38340	3-1	SALK_144950	5'UTR	member of the DREB subfamily A-2 of ERF/AP2 TF family
At5g41570	5-1	SALK_119740	exon	WRKY family transcription factor (WRKY24)
	5-4	SALK_008183	exon	
At5g46830	8-3	SALK_060048	exon	basic helix-loop-helix (bHLH) family protein (AtbHLH028, ERF2)
At1g34670	9-1	SALK_131752	exon	MYB family transcription factor (AtMYB93)
At1g01720	13-2	SALK_067648	exon	transcriptional activator with NAC domain (ATAF1)
At4g33960	17-1	SALK_149207	exon	expressed protein, homology to MADS-box

2.2.2 Seed sterilisation

To avoid disturbing fungi and bacterial colonies either on plates (see chapter 2.2.2) or on hydroponics (see chapter 2.2.5), seeds were sterilized. Microorganisms were eliminated by adding 70% ethanol for 2 minutes and 3% sodium hypo chloride (NaClO) with one drop of Triton X100 for next 15 minutes, whereby plant seeds with their thick out layer was not damaged. The NaClO/Triton X100 solution was removed by carefully pipetting and seeds were washed with sterile water 3 to 5 times. After removing the water seeds were or air-dried or resuspended in 0.1% sterile agarose for imbibition. All steps occurred under sterile conditions.

2.2.3 Sterile liquid cultures

Wild-type Col-0 seedlings (100-120 seeds) were grown in 30 ml of sterile liquid FN medium or 150µM SO_4^{2-} medium (250 ml Erlenmeyer glass flasks) on orbital shakers with constant, uniform fluorescent light (~50 µE in the flask) and temperature (22°C). Shaker speed was low (30 rpm) during the first three days, and then increased to 80 rpm. Care was taken to prevent significant clumping of seedlings. After seven days the FN media was replaced with another 30 ml of fresh FN medium, whereas the 150µM SO_4^{2-} medium was replaced with 30 ml of low sulphate (-S) medium, in which plants were subjected for sulphur deprivation for 2 next days.

Full nutrition media provided the seedlings sufficient sulphate and thereby was used as a control. The 150μM sulphate in the medium allowed seedlings to germinate and grow but after 7 days of cultivation there was no excess of the sulphate ions which could be stored in plant's vacuoles, assuring S-starvation during two days.

On day 9 FN cultures and some of the –S cultures were harvested. At the same time all other flasks of S-starved cultures were opened, and re-closed either without addition or after addition of 1ml 15 mM K_2SO_4 (500μM final concentration) or 1 ml 15 mM KCl (500μM, control).

Cultures re-supplied with sulphate ions (or KCl) were harvested after 12 min, 30 min and 3 hours. Plant material from each flask was quickly (<10 sec for the entire procedure) blotted on tissue paper, washed twice in an excess of deionised water, blotted on tissue paper again and frozen in liquid nitrogen (LN_2). Materials were stored in (LN_2) until pulverization using mortar and pestle. Ground material was stored at –80° C until further use.

Table 2-3 Sterile full nutrition and low-sulphate medium composition

	Full nutrition (FN)	150μM SO_4	Low sulphate (-S)
Compound	**Final [mM]**	**Final [mM]**	**Final [mM]**
KNO_3	2	2	0,1
NH_4NO_3	1	1	0,05
KH_2PO_4/K_2HPO_4 (pH 5,8)	3	3	3
$CaCl_2$	4	4	4
$MgSO_4$	1	0,15	0
K_2SO_4	2	0	0
$MgCl_2$	0	0,85	1
KCl	0	4	4
MES (pH5,8)	3	3	3
Microelements	1x *	1x *	1x *
Sucrose	0,5%	0,5%	0,5%
Glutamine	1	1	1

*Microelements: 40μM $Na_2FeEDTA$, 60μM H_3BO_3, 14μM $MnSO_4$, 1μM $ZnSO_4$, 0,6μM $CuSO_4$, 0,4μM $NiCl_2$, 0,3μM $HMoO_4$, 20nM $CoCl_2$

2.2.4 Growth on agar plates

To imbibe *Arabidopsis* (Col-0) wild-type seeds, they were kept in sterile 0,1% agar in darkness of 4° C for 3-4 days. Plants were then grown on half-strength Murashige and Skoog medium (half MS) (Murashige and Skoog, 1962), supplemented with 1% (w/v) sucrose and solidified with 0,7% agar at 22° C under a 16 h day (140 μmol m^{-2} s^{-1}) - 8h night regime. Composition of half MS medium was modified when applying for plant selection or root architecture studies (see chapter 2.2.2.3) performed on normal S and low S media.

2.2.4.1 Selection on BASTA-plates

To select the *Arabidopsis* transformants resistant to herbicide BASTA, the sterile seeds were sown on the plates with the half MS medium, us described above (chapter 2.2.4), with phosphinotricine (PPT; the active substance in a number of effective and environmentally friendly herbicides such as Basta; another name: glufosinate) in a final concentration 20 µg/ml. Seeds were germinated and seedlings were grown at 22° C under constant light (200-250 µmol m^{-2} s^{-1}) until it was possible to distinguish between healthy, green, homo- or heterozygous and yellow (wild type-like) seedlings. The survivors were than replanted to target conditions like i.e. another agar medium or hydro culture (see chapter 2.2.5).

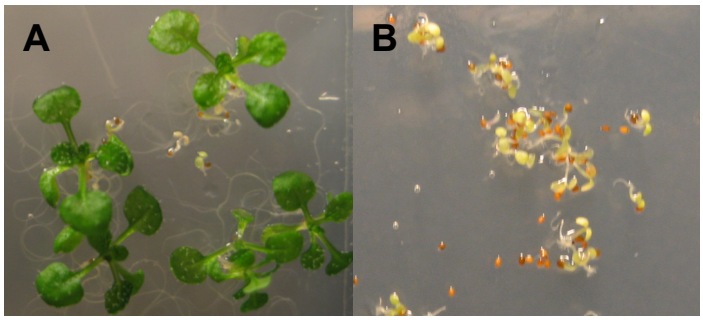


Fig 2-1 Selection on BASTA-plates. The resistant to BASTA *Arabidopsis* transformants remain healthy, vigorous and easily distinguishable from wild type-like, yellow plants (A). Panel B presents Col-0 plants as a control.

2.2.4.2 Selection on canamicin-plates

The integrated T-DNA contained a canamicin resistance gene. Screening for positive integrations was done by addition of 50 µg/ml canamicin to the half MS medium, prepared us described above (chapter 2.2.4). About 50 sterilized and dry seeds were spread onto an agar plate, containing half MS media with canamicin. Plates were deposited in a climate chamber with constant conditions (temperature: 22°C, constant light, light intensity: 200-250 µmol m^{-2} s^{-1}, air humidity 50%) for two weeks. Plate preparation and seed application occurred under sterile conditions.

2.2.4.3 Vertical plates

All OX lines and all available homozygous knock out (KO) lines were grown vertically on square plates for the root architecture studies. The plates were filled with 0,5 MS media supplemented with 1% (w/v) sucrose and modified to have one of 4 different sulphate concentrations: 750 µM SO_4^{2-} (control), 50 µM SO_4^{2-} and 20 µM SO_4^{2-} for low S conditions and zero sulphate. In all media, the –S micro elements were used. In macro elements the sources of SO_4^{2-} ions were substituted by their chlorides, in same molarity (see table 2-4). The agar content was increased up to 1% (v/v).

6 to 7 sterilized seeds were set in a line in the upper quarter of the plate, always parallel to the WT on the same plate. Afterwards the plates were sealed, transferred to a climate chamber (temperature: 22° C, constant light, light intensity: 200-250 µmol m^{-2} s^{-1}, air humidity 50 %) and stored

vertically in a plastic box to protect growing roots from the light (light could only reach the upper quarter of the plate, where the shoots were growing). Plants were grown for 16 days in total. Root length was checked every second day. On the 6th, 8th, 11th, 13th, 14th ad 22nd (depending on experiment) day the root length and architecture was photographically documented.

Table 2-4 Half-strength Murashige and Skoog medium composition; manipulated compounds depicted in bold.

Compound	**Normal half MS**	**50µM SO4 MS**	**20µM SO4 MS**	**0µM SO4 MS**
Macro elements	mM	mM	mM	mM
NH4NO3	10,305	10,305	10,305	10,305
KH2PO4	0,625	0,625	0,625	0,625
MgSO4	**0,75**	**0,05**	**0,02**	**0**
MgCl2	**0**	**0,7**	**0,73**	**0,75**
KNO3	9,395	9,395	9,395	9,395
CaCl2	1,495	1,495	1,495	1,495
Micro elements	µM	µM	µM	µM
CuCl2	**0,05**	**0,05**	**0,05**	**0,05**
ZnCl2	**14,955**	**14,955**	**14,955**	**14,955**
MnCl2	**0,05**	**0,05**	**0,05**	**0,05**
H3BO3	0,05	0,05	0,05	0,05
CoCl2	0,055	0,055	0,055	0,055
KI	2,5	2,5	2,5	2,5
Na2MoO4	0,515	0,515	0,515	0,515
Fe-EDTA	100 µM	100 µM	100 µM	100 µM
Vitamines	mg/l	mg/l	mg/l	mg/l
Nicotinic acid	0,25	0,25	0,25	0,25
pyridoxine	0,25	0,25	0,25	0,25
thiamine	0,05	0,05	0,05	0,05
glycine	1,00	1,00	1,00	1,00
Myo-inositol	50,00	50,00	50,00	50,00

2.2.5 Hydroponic system

Access to root material for analyses is in most cases limiting. To yield sufficient material hydroponic cultivation of plants is a convenient method. It allows studies on root morphology and architecture in control or e.g. different nutritional regimes. Furthermore, nutrients can be changed within seconds. Another advantage in comparison to soil grown plants is the harvesting of intact, clean whole roots to perform experiments on this material.

The hydroponic system consisted of autoclaved 1000 µl pipette-tips boxes (Eppendorf) filled with 0,5 l of medium based on Hoagland. The composition of micro- and macro elements is described in table 2-5. Two different conditions, sulphur sufficient (normal S) and sulphur deficient (–S), were applied. In case of –S medium, magnesium sulphate was replaced with magnesium nitrate at the same concentration. However, the low S medium contained still 1,8% residual sulphate originating from the micronutrients and the amount of nitrate in –S medium was slightly (3,75%) increased.

Arabidopsis plants transformed with 35S constructs (possessing the TF gene sequence or transformed with empty vector) were pre-grown on BASTA-agar plates for around 3 weeks. In parallel the Col-0 plates were cultivated on agar plates without BASTA. After this period BASTA-resistant transformants and Col-0 plants were picked up and placed directly on the Eppendorf-box, dipping the roots in respective medium. Boxes were kept covered with the transparent cover for one week to allow plants slowly adjust to the lower-humidity greenhouse conditions, when compared to the high humidity inside the agar plates. The hydro cultures were grown for the next 2-3 weeks. After that period the plants were big enough either to perform nutritional experiments or to be harvested.

Table 2-5 Composition of the used hydroponic media, based on Hoagland; for sulphur deficient conditions magnesium sulphate was replaced with magnesium nitrate

Compound	**Concentration**
Macro elements	[mM]
Ca(NO3)2	2
KH2PO4	0,5
MgSO4	0,75
KNO3	10
Micro elements	[µM]
CuSO4	1,5
ZnSO4	2
MnSO4	10
H3BO3	50
MoO3	0,1
KCl	50
Fe-EDTA	50

All media were changed weekly. The boxes were carefully rinsed with fresh medium and filled up with –S medium for starved plants or refilled with normal medium for control plants, to treat all plants the same way.

All 35S lines were deprived for sulphur for 10 days. Root and leaf material of hydro culture was harvested separately, collected quickly, rinsed with ultra pure water and dried with a paper towel before weighing and freezing in the liquid nitrogen. Material was ground under liquid nitrogen with a mortar and pestle and stored at -70°C.

2.2.6 Growth on soil

Plate-grown, 14-days-old *Arabidopsis* seedlings were picked to GS90 soil: vermiculite mixture (1:1 v/v) (Fritz Kausek GmbH & Co. KG, Mittenwalde, Germany) and grown in the glasshouse under the following conditions: 16h day with 21°C and 8h night with 17°C, relative humidity 50%. Plants were watered with tap-water every second day for six weeks. Plants were harvested into paper bags and dried for 2 weeks to obtain seeds. Seeds were harvested in 2 mL screw-cup glass vials and stored at 14°C and 10% relative humidity.

2.3 Methods of molecular biology

2.3.1 RNA isolation procedures

2.3.1.1 TRIzol maxi-prep protocol

Total ribonucleic acid (RNA) was isolated from shoots or roots using TRIZOL reagent (Invitrogen),(http://www.*Arabidopsis*.org/info/2010_projects/comp_proj/AFGC/RevisedAFGC/site2Rna; Chomczynski and Sacchi, 1987). RNA concentration was estimated by measuring A_{260} in a spectrophotometer (Eppendorf) and applying the formula: [RNA] = A_{260} x D x 40µg/µL, where D is the RNA dilution factor. RNA quality was judged from $A_{260/280}$ (ratio 1,8-2,0 indicates low protein contamination) and $A_{260/230}$ (ratio ≥ 2,0 indicating low polysaccharide contamination). To remove all traces of DNA contamination, 200 µg of total RNA was digested with Dnase I RNase-free (Sigma), according to the manufacturer's instructions. Absence of genomic DNA contamination was subsequently confirmed by PCR, using primers designed on an intron sequence of a control gene: *At5g65080* (primer sequences in Appendix E). RNA integrity was checked on a 1,5% (w/v) agarose gel both prior to, and after DNaseI digestion. Poly-A^+ RNA was purified with an Oligotex mRNA Mini Kit (Qiagen) using the supplier's batch protocol.

2.3.1.2 RNA extraction using TRIzol mini-prep protocol

Frozen plant material (100-200 mg) was ground in LN_2 in pre-cooled mortar or homogenised using metal beads (Ø 5mm) in the MM200 homogeniser (Retsch). TRIzol reagent (Invitrogen) was then added and mixed well by vortexing. After 5 min incubation at room temperature the homogenate was centrifuged at 13000 x g for 5 min at 4°C in pre-cooled centrifuge. The supernatant was removed to a fresh Eppendorf tube and 400µl of chloroform was added and mixed by vortexing before incubation at RT for 5 min. After 10 min centrifugation at 13000 x g, at 4°C, the aqueous phase (~1 mL) was transferred to a fresh Eppendorf tube. RNA was precipitated with 0,5 ml of isopropanol and 0,5 ml of HSS buffer (0,8 M Sodium Citrate and 1,2 M NaCl) per 1 ml of aqueous phase, overnight at –20°C. The precipitate was pelleted by centrifugation at 13000 rpm for 30 minutes at 4°C. The supernatant was removed and the pellet was washed twice with 1 ml 70% EtOH, air dried, and re-suspended in ca. 50 µl water (approx. 1µg RNA/ 1µl). RNA amount and purity was determined by photometric measurements at 230, 260 and 280nm (see above).

2.3.2 cDNA synthesis

Reverse transcription reactions were performed using 500 ng of poly-A^+ RNA or 5µg of total RNA, with SuperScript™ III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The efficiency of cDNA synthesis was assessed by real-time PCR amplification of control genes encoding *ubiquitin10* and *GAPDH* (primer sequences in Appendix E). Only cDNA preparations that yielded similar C_T values (e.g. 20±1) for the control genes were used for subsequent comparison.

2.3.3 Real time PCR conditions and analysis

PCR reactions were performed in an optical 384-well plate with an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems), using SYBR® Green to monitor dsDNA synthesis. Reactions contained 5 µl 2X SYBR® Green Master Mix reagent (Applied Biosystems), 1 ng cDNA and 200 nM of each gene-specific primer in a final volume of 10 µl. A master mix of sufficient cDNA and 2X SYBR® Green reagent was prepared prior to dispensing into individual wells, to reduce pipetting errors and ensure that each reaction contained an equal amount of cDNA. An electronic MultiPro™ Pipette (Eppendorf) was used to pipette the cDNA-containing master mix, while primers were aliquoted with an Eppendorf 12-channel pipette. Reactions were also scaled-down to 5 µl, containing 2,5 µl of 2X SYBR® Green Master Mix reagent (Applied Biosystems), 0,5 µl of cDNA and 2 µl of each gene-specific primer (200 nM final concentration of each primer). An Evolution P3 liquid handling system (Perkin Elmer) was used for the 5 µl reactions set-up on 384 well plates.

The following standard thermal profile was used for all PCR reactions: 50° C for 2 min; 95° C for 10 min; 40 cycles of 95° C for 15 sec and 60° C for 1 min. Data were analysed using the ABI SDS 2.2 software (Applied Biosystems). To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔR_n) versus cycle number, baseline data were collected between cycles 3 and 15. All amplification plots were analysed with an R_n threshold of 0,2 to obtain C_T (threshold cycle) values. In order to compare data from different PCR runs or cDNA samples, C_T values for all TF genes were normalised to the C_T value of *ubiquitin10*, which was the most constant of five house-keeping genes (*actin*2, *ubiquitin10, β-6-tubulin, elongation factor 1 alpha, adenosyl-phosphoribosyltransferase*) included in each PCR run. The average C_T value for *ubiquitin10* was 17,8 (+/- 0,47) for all plates/templates measured in this series of experiments. PCR efficiency (E) was estimated by the method, which made use of data obtained from the exponential phase of each individual amplification plot and the equation $(1+E) = 10^{slope}$ (Ramakers et al., 2003). TF gene expression was normalised to that of *ubiquitin10* by subtracting the C_T value of *ubiquitin10* from the C_T value of the TF gene of interest. Expression ratios of sample A to sample B were then obtained from the equation $(1+E)^{-\Delta\Delta CT}$ where $\Delta\Delta C_T$ represents ΔC_{TA} minus ΔC_{TB}, and E is the PCR reaction efficiency. Dissociation curves of the PCR products were analysed using ABI SDS 2.2 software.

2.3.3.1 Real time reversed transcription (RT)-PCR-based platform

To enable quantitative measurement of transcripts for 1465 *Arabidopsis* TFs, the real time reversed transcription (RT)-PCR-based platform was developed by the Molecular Genomics and Molecular Plant Nutrition Groups (MPI-MP, Golm, Germany). Putative TF genes were identified in the *Arabidopsis thaliana* genome by taking advantage of gene annotations and INTERPRO domain searches (Riechmann and Ratcliffe, 2000) at the MIPS (http://mips.gsf.de/cgi-bin/proj/thal/) TAIR (http://www.arabidopsis.org) and AGRIS (http://arabidopsis.med.ohio-state.edu/AtTFDB/) databases. The resulting set of sequences was supplemented by performing BLASTP and TBLASTN searches (http://www.ncbi.nlm.nih.gov/blast/), to uncover further possible TF genes in the *Arabidopsis* genome.

Primers were designed according to a stringent set of criteria as described by Czechowski et al. (2004). The sequences of each primer pair are given in Czechowski et al. (2004). The library of 1465 primer pairs was arrayed on 4 and ¼ 384-well plates and this stock, used together with SYBR® Green and ABI PRISM® 7900HT PCR system, allowed one researcher to measure expression of the whole set of TFs in a single biological sample in a just one working day (Czechowski et al., 2004).

2.3.3.2 Real-time PCR primer design

All primer sequences for non-transcription factor genes (primer sequences in Appendix E) were designed following the same stringent set of criteria as described in Czechowski et al. (2004). They were designed using Primer Express 2.0 software (Applied Biosystems) with the following parameters: melting temperatures (Tm) of 60±2ºC, primer lengths of 20-24 nucleotides, guanine-cytosine (GC) contents of 45-55%, and PCR amplicon lengths of 60-150 base pairs. In addition, when possible at least one primer of a pair was designed to cover an exon-exon junction, according to the gene structure models at TAIR (http://www.arabidopsis.org). Where possible, primers were designed close (no more than 500 bp) from the 3' end of longest gene transcript annotated in TAIR (www.arabidopsis.org) and primer sequences were blasted against the *Arabidopsis* genome sequence using TAIR BLAST (http://www.arabidopsis.org/Blast/) with standard parameters to check their specificity. All the qRT-PCR measurements performed with non-transcription factor gene primers were curried out under the same standard set of reaction conditions, described above (chapter 2.3.3).

2.3.4 DNA isolation

For PCR-based screening of homozygous SALK knockout lines, the total DNA was extracted with the Extract-N-Amp Plant PCR kit (Sigma). The two-step extraction procedure was done according to the manual using one whole and untreated frozen rosette leaf.

2.3.5 PCR – based screening for homozygous knock-out (KO) lines

Two pairs of primers were used to identify homozygous KO lines: two gene specific primers unable to amplify product of expected size from homozygous KO and a gene specific primer plus a T-DNA specific primers (primer sequences in the Appendix E), that amplify DNA only from a KO lines but not from the WT. T-DNA specific primers were designed on the sequence of the vectors used to create the mutant lines. All gene-specific primers were designed using web-based software provided by SIGnAL (http://signal.salk.edu/tdnaprimers.html) with the following parameters: optimal primers size - 21bp; optimal T_m - 65° C; GC content between 20 and 80%; maximum distance from the insertion site – 10 bp. PCR was performed on DNA prepared by the Extract-N-Amp Plant PCR kit (Sigma), as described above, using the combination of two gene specific primers or one gene specific primers and primer LBb1 (primer sequences in Appendix E). Genomic DNA from WT grown in parallel was always used as control in both PCR reactions. PCR products were visualised by EtBr staining following electrophoresis on agarose gels.

The most commonly used touch down PCR program for PCR-based screening was:

1 cycle of 94° C for 2 min.,

2 cycles of 94° C for 1 min.; (T_m primers +4) °C for 1 min., 72° C for 1min.,

2 cycles of 94° C for 1 min.; (T_m primers + 3) °C for 1 min., 72° C for 1min.,

24 cycles of 94° C for 1 min.; (T_m primers -2) °C for 1 min., 72° C for 1min.,

1 cycle of 72° C for 10min.

2.3.6 DNA cloning

The gain of function approach involved cloning of selected TF genes into binary p-Green vector for constitutive over expression driven by 35S promoter. This part of the work was done by Dr Isabell Witt and Dr Maria Ines Zanor (then members of Plant Signaling Group, MPI-MP, Golm, Germany), following the standardized cloning procedure as describes in Skirycz et al., (2006).

2.3.7 Plant transformations

Transformation of *Arabidopis thaliana* Col-0 with *Agrobacterium tumefaciens* was performed using the floral dip method (Clough and Bent, 1998).

2.3.8 RNA gel blot analysis

RNA gel blot analysis was performed as described by Gomez-Merino et al. (2004).

2.3.9 Selection of over expressing lines using Northern blot hybridisation

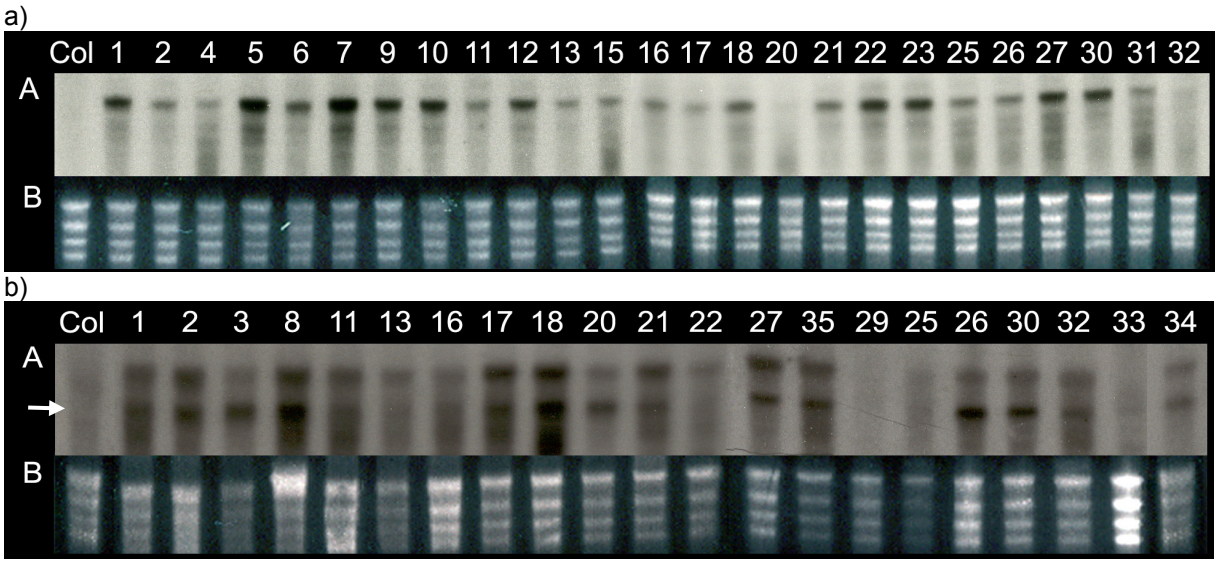


Fig. 2-2 Northern blot analysis of plants constitutively over expressing TF genes: At2g28550 (line 63) **a)** and At5g60120 (line 70) **b)**. On figure b the lower band corresponds to the specific DNA fragment and is depicted by white arrow. On both figures the A panel shows the hybridization blot and panel B shows the RNA electrophoresis gel image.

RNA was isolated from transformed T1 plants containing constitutive constructs of the AP2 TF genes: *35S-At2g28550* and *35S-At5g60120* and subjected to Northern blot analysis using radioactive isotope P^{32}. (Fig 2-2). RNA prepared from soil grown Col-0 was always run in parallel for control. Typically, endogenous expression level of target TF genes was below the detection limit of Northern blots (Fig 2-2, lines "Col"), while transgene expression driven by the 35S promoter was clearly detected on such blots.

Plants number 5, 7, 10, 12 and 30 for the line 63 (containing *35S-At2g28550* construct) and plants 3, 8, 18, 20, 26, 30 for the line 70 (containing *35S-At5g60120* construct) were selected for further analysis.

T2 generation seeds were harvested from all selected T1 over expressing *Arabidopsis thaliana* plants and kindly provided by co-workers (see chapter 2.2.1.2).

2.3.10 Transcription profiling on Full Genome Chip

ATH1 (Affymetrix, 22,800 genes of *Arabidopsis thaliana*, each represented by 11 oligonucleotides and mismatches) was used for transcript level observation in order to perform a genome wide analysis. For hybridization of the ATH1 chips, 50 µg of clean and intact RNA from each sample of sterile liquid cultures plant material (see chapter 2.2.3) was sent together with the ATH1chips to German Resource Center for Genome Research (RZPD, Berlin, Germany).

2.4 Biochemical techniques

2.4.1 HPLC – high performance liquid chromatography

2.4.1.1 Determination and measurement of thiol-group containing compounds derivatised with monobromobimane

Around 100 mg of the powdered frozen plant material was used for the 1:5 extraction of thiols with 0,1M HCl. Around 30 mg of polyvinylpolypyrrolidone (PVPP) was added immediately to the extraction mixture. The PVPP had been washed before with 0,1 N HCl and dried. The extraction was done under shaking with 500 rpm for 40 min at RT. After centrifugation for 15 min at 4° C and 14000 rpm the supernatant was removed and either analyzed immediately or stored at -20° C.

Extracted thiols were first transferred quantitatively into their reduced form. Reduction step was implemented with the addition of 70 µl freshly prepared 10 mM DTT to 120 µl of the extract with 200 µl of 0,25 M N-cyclohexyl-2-aminoethanesulphonic acid (CHES) buffer (pH 9,4 with sodium hydroxide [NaOH]). The sample mix was incubated for 40 min at RT.

A direct measurement of thiol-containing compounds (cysteine, γ-glutamylcysteine [γ-EC], glutathione and homocysteine) is not possible. Thus, the reduced thiols were transferred into

fluorescently active derivatives, by the fluorescent dye monobromobimane (mBrB, 3-bromomethyl-5-ethyl-2,6-dimethyl-pyrazolo[1,2-α]pyrazol-1,7-dione, Calbiochem). The reaction is depicted in Fig. 2-2.

Derivatisation was curried out during incubation with 10 µl 25 mM mBrB in acetonitrile for 15 min at RT in the dark, as this substance is photosensitive. The reaction was stopped by addition of 220 µl 15% HCl and another incubation for 30 min at 4° C in the dark. After spinning down the cell debris for 20 min at 4° C and 14000 rpm, the supernatant was transferred into HPLC glass vials with lids and either stored at 4° C in the dark or directly measured.

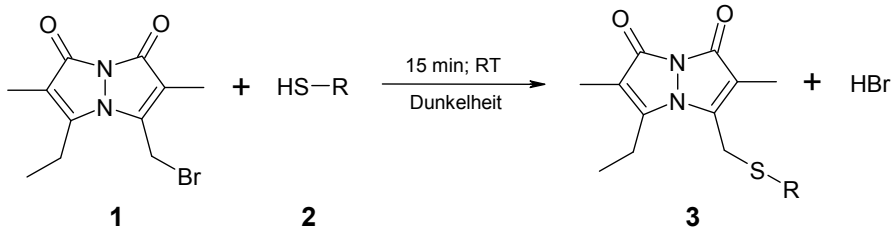


Fig. 2-3 Derivatisation of thiol group containing compounds with mBrB. **1**=monobromobiman (mBrB), **2**=thiol-containing compound, **3**=fluorescently active mBrB-derivative

Labeled thiols were separated chromatographically by reversed phase chromatography (RP-HPLC) (according to Fahey et al., 1981) and sensed by a fluorescent detector. 30 µl of derivatised extract was applied to the octadecyl silicate column (25 cm in length and 4 mm in diameter with a grain size of 5 µm, Knauer) and separated by an increasing gradient of hydrophobicity (as described in table 2-7) with a flow rate of 1 ml/min. The hydrophobicity gradient was obtained by mixing two different elution solvents (see table 2-6). The whole separation step for thiol-compounds took 35 min, whereby the thiols were eluted between the 13th and 19th minute (figure 2-4).

Table 2-6 Composition of elution solvents for thiol-containing compounds separation on HPLC.

elution solvent	methanol [%; v/v]	acetic acid [%; v/v]	pH
A	10	0,25	3,9
B	90	0,25	3,9

Table 2-7 Elution protocol for thiols; the composition of the current elution solutions is described in %B of A, at the time in min; flow and temperature are constant.

time [min]	B [%]	flow [ml/min]	temp [°C]
0	0	1	25
2.0	0	1	25
12.0	8	1	25
17.0	14	1	25
19.0	100	1	25
30.0	100	1	25
31.5	0	1	25
35.0	0	1	25

The fluorescence measurement of mBrB derivatives occurred at 480 nm emission wavelength under light excitation of 380 nm. Additional to the samples, thiol standard solutions containing L-cys, γ-EC, GSH and homocysteine (hcy) were measured.

All buffers were degassed before applying to the column. In order to filter out the contaminations, the 10 mm pre-column filled with Hypersil ODS (Supelco) was used.

The HPLC-system was operated by the Chromeleon 6.30 chromatography data software (Dionex), which was also used for data evaluation.

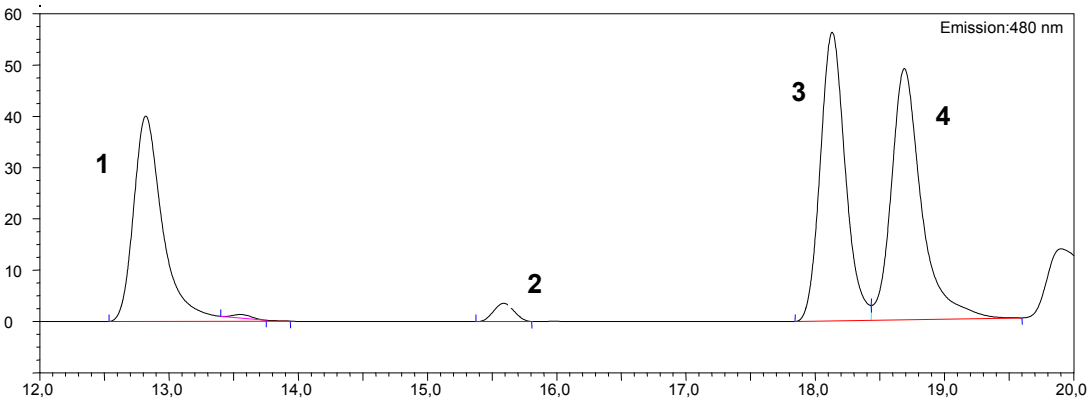


Fig. 2-4 RP-HPLC chromatogram of mBrB derivatised thiol compounds : retention time of the measured thiols and standards substance in min; **1**=Cys, **2**=γ-EC, **3**=GSH, **4**=Hcy

2.4.1.2 Determination and measurement of free amino acids derivatised with ortho-phthaldialdehyde

Amino acids were derivatized with ortho-phthaldialdehyde (OPA), a fluerescence dye (Lindroth and Mopper, 1979; Kreft et al., 2003), to enable their detection. OPA reacts at an alkaline pH value and in presence of mercapto-group containing compounds with primary amines to fluorescently active isoindole derivates, as depicted in figure 2-3. All proteinogenic amino acids, except proline (pro, no primary amino group) and cysteine (no clear labeling because of instability and formation of cystine) were detected. Cysteine was observed as described in the previous chapter by derivatisation with mBrB.

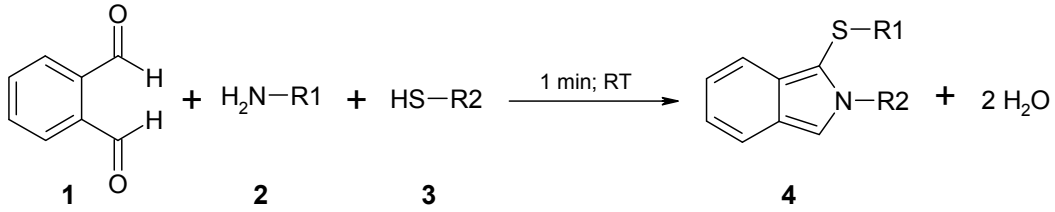


Fig. 2-5 Derivatisation of amine group containing compounds with OPA; **1**=ortho-phthaldialdehyde (OPA), **2**=amino acid, **3**=mercapto-group containing compound, **4**=fluorescently active isoindole derivate

Amino acids were extracted through a multiple step procedure with N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulphonic) acid (HEPES) and different ethanol concentrations (according to Scheible et al., 1997). 400 µl 80% ethanol in 2.5 mM HEPES (pH 7.5 with KOH) were added to 100

mg of ground frozen plant material and the mixture was shaken for 20 min at 80° C with 500 rpm. After centrifugation for 10 min at 4° C and 14000 rpm the supernatant was removed and re-extracted with 400 µl 50% ethanol in 2.5 mM HEPES (pH 7.5) and shaken for 20 min at 80° C with 500 rpm. After another centrifugation for 10 min at 4° C and 14000 rpm the supernatant was removed and for a third time extracted with 200 µl 80% ethanol in 2,5 mM HEPES (pH 7.5) again for 20 min at 80° C with 500 rpm. The extraction mix was centrifuged a last time for 10 min at 4° C and 14000 rpm and the supernatant was taken and either immediately analyzed or stored at -20° C.

Table 2-8 Elution protocol, describing the composition of the current elution solution in %B of A at the time in min

time [min]	B [%]	flow [ml/min]	temp [°C]
0	0	0,8	30
2,00	0	0,8	30
16,00	13	0,8	30
23,25	15	0,8	30
32,30	50	0,8	30
43,30	60	0,8	30
49,30	100	0,8	30
51,30	100	0,8	30
58,30	0	0,8	30
60,00	0	0,8	30

All extracted amino acids were mixed with 0,2 volume with 0,8 M borate buffer (pH 10,4, Crom Analytic) just before the measurement procedure and then filled into the HPLC glass vials with lids. Next 70 µl of the sample volume was mixed in the ratio 1:1 with the OPA-derivation reagent, which was composed of 0,5% (w/v) OPA in 0.7 M borate buffer with 10% (v/v) ethanol and 1% (v/v) β-mercaptoethanol. The incubation time was 1 min, directly performed in the injection sampler to achieve reproducible results.

Table 2-9 Composition of elution solvents for amino acid measurement elution solvent

elution solvent	tetra hydrofurane [%; v/v]	methanol [%; v/v]	acetonitrile [%; v/v]	sodium phosphate buffer [mM]	pH
A	0,2	0	0	8,5	6,8
B	0	32,5	20,5	18,5	6,8

For separation of amino acids, an RP-column, 12 cm in length and 4,6 mm in diameter with a grain size of 3 µm and octadecyl silicate as stationary phase (Knauer), was used. 15 µl of the OPA-labeled sample mix were injected and separated by a none linear gradient of two different buffers as described in table 2-9. The buffers differed in their hydrophobicity and composition as illustrated in table 2-8 and were pumped with a flow rate of 0,8 ml/min. The fluorescence detection of the OPA-labeled amino acid derivatives was detected at 450 nm emission wavelength and an excitation wavelength of 330 nm. In total 65 min were required for a regular separation of amino acids. Aspartic acid (asp), eluted firstly in the 2nd minute, the last amino acid was lysine (lys) in the 54th minute.

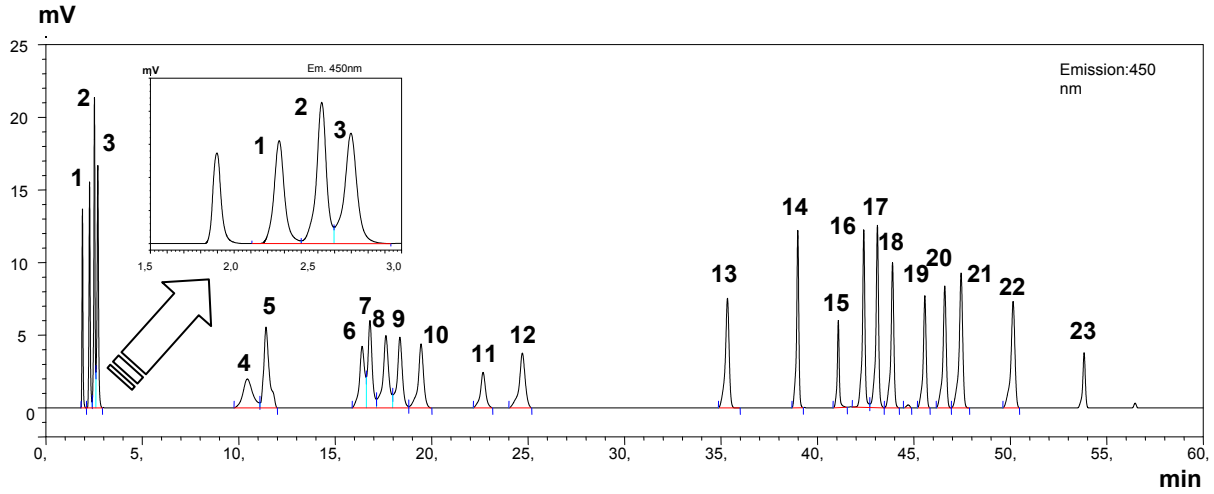


Fig. 2-6 RP-HPLC chromatogram of OPA-derivatised amino acids; retention time of the measured thiols and standards substance in min; **1**=O-Phosphohomoserine (OPHS), **2**=Asp, **3**=Glu, **4**=Asn, **5**=Ser, **6**=S-Methylmethionine (SMM), **7**=Gln, **8**=Gly, **9**=Homoserine (Hse), **10**=Thr, **11**=His, **12**=Ala, **13**=Arg, **14**=Tyr, **15**=Cystathionine, **16**=Val, **17**=Met, **18**=Norvaline, **19**=Trp, **20**=Phe, **21**=Ile, **22**=Leu, **23**=Lys.

After a short time of elution with buffer B the separation step was completed and the column cleaned from any substance, the concentration was lowered to zero in order to prepare the column for the next measurement. For amino acid quantification the following standard solutions in a range of 150-3000 pmol were used for amount determination: asp, glutamate (glu), asparagine (asn), ser, SMM, glutamine (gln), glycine (gly), homoserine (hse), threonine (thr), histidine (his), alanine (ala), arginine (arg), tyrosine (tyr), cystathionine (cst), valine (val), met, norvaline (nor-val), tryptophan (trp), phenylalanine (phe), isoleucine (ile), leucine (leu) and lys.

2.4.1.3 Determination and measurement of polyamines through their dansyl derivatives

RP-HPLC in combination with fluorescence spectrophotometry was used to separate and quantitate polyamines (putrescine, spermidine and spermine) through their dansyl derivatives, as it is the most sensitive polyamines detection method (Smith and Davies, 1985).

100 mg of the powdered frozen plant material was used for the extraction of polyamines with 1 ml of 0,2 M perchloric acid (PCA; $HClO_4$). After incubation for 1h at 4° C the homogenates were centrifuged for 30 min at 4° C and 14000 rpm. The supernatant and pellet were collected separately. The supernatant was used to determine PCA-soluble free (fraction 1) and PCA-soluble conjugated polyamines (fraction 2), whereas the pellet was used to determine PCA-insoluble bound polyamines (fraction 3).

Free PCA-soluble fraction could undergo the dansylation process directly, while the PCA-soluble conjugated fraction had to be processed via acidic hydrolysis, prior to dansylation. The pellet was hydrolysed by adding 200 µl of 37% HCl and incubation at 110° C for 18-20h. Afterwards, HCl was evaporated from the tube by heating at 70° C. The residue was than resuspended in 200 µl of 0,2 M $HClO_4$, which made it ready for the dansylation.

To extract PCA-insoluble bound polyamines, the pellet was rinsed two times with 1ml of 0,2 N PCA to remove any trace of soluble polyamine and then dissolved by vigorous vortexing in 200 µl of 1 N NaOH. The mixture was sonicated for 90 min. The next step, acidic hydrolysis, was performed in the same way as described above.

The dansylation was done according to the methods of Flores and Galston (1982). 100 µl aliquots of each fraction were added to 10 µl of 0,5 M diaminohexan (internal standard), 110 µl (1 volume) of 1,5M sodium carbonate (Na_2CO_3) and 200 µl dansyl chloride in acetone (7,5 mg/ml). The mixture was incubated at 60° C for 1h in the dark. 50 µl of proline (100 mg/ml) was added to the mixture to saturate excessive dansyl chloride. After further incubation for 30 min at 60° C in the dark, the polyamines were extracted with 250 µl of toluene and vigorous vortexing for 30 sec. The mixture separated into two phases, aquatic and organic. The organic, upper phase, containing polyamines, was collected and dried in speedvac. The polyamine residue was dissolved in 100 µl of methanol (Lichrosolv) and assayed immediately or stored (no more than 1 week) at -20° C in the dark.

20 µl of a sample were injected onto a reverse phase LC-18 column protected by a guard column (Alphabon C18, 10 µm; Supelco, Germany). Samples were eluted from the column with a solvent gradient (v/v) of water: methanol changing from 70% to 100% in 15 min at a flow rate of 1 ml/min. (70-80% methanol for 5 min, 80-100% methanol for 10 min, 100-70% methanol for 5 min). Elution was completed after 25 min.

Eluates from the column were detected by an attached fluorescence detector (RF 2000, Dionex). For the dansylated polyamines, an excitation wavelength of 365 nm was used with an emission wavelength of 519 nm. Under these conditions, retention times of putrescine, diaminohexan, spermidine and spermine were 9,340 $\pm$0,500, 10,560 $\pm$0,500, 13,320 $\pm$0,500 and 16,140 $\pm$0,500 min, respectively. The HPLC-system was operated by the Chromeleon 6.30 chromatography data software (Dionex), which was also used for data evaluation. Peak areas were integrated by an integrator and concentrations were calculated according to a calibration curve of known polyamines.

2.4.2 Ion chromatography

Free ions (sulphate, nitrate and phosphate) were separated and quantified by The Dionex ICS-2000 Ion Chromatography System (ICS-2000), which performs an ion analyses using suppressed conductivity detection.

Around 50 mg of the powdered frozen plant material was used for the 1:5 extraction of ions with 0,1mM HCl. After vigorous vortexing, the samples were centrifuged for 5 min at 14000 rpm at 4° C. The supernatant was collected, centrifuged again and the second supernatant was filtered through the Ultrafree MC 5000 NMWL Filter Unit (Milipore) at 5000g at 4° C. The samples were stored in -20° C or measured immediately after adjusting the ions concentration range by 1:20 dilution with Millipore water.

An ion chromatography system consisted of a liquid eluent, a high-pressure pump, a sample injector, a guard and separator column, a chemical suppressor, a conductivity cell and a data collection system. The ICS-2000 included an eluent generator, which provided a gradient delivery by

mixing two elution solvents: water and KOH. The eluent gradient was increasing over each sample measurement up to 23 mM KOH. For the maximum eluent gradient the suppressor current was 20 mA, which allowed the suppressor to remove the ions generated from the eluent itself and therefore to enhance the sample ions detection selectively.

15 µl of the sample was automatically injected into the eluent stream and was pushed by pump through the guard (for removing the contaminants) and the separator columns (filled with a polymeric resin as a stationary phase). The different ions were separated via ion exchange, based on their different interactions with the ion exchange sites and thus their different migration rates through the IC column. The temperature for separator column and the conductivity cell was 30° C. The conductivity cell transmitted the electrical conductance signal of each ion sample to Chromeleon data collection system. Standard solutions were used to calibrate the ion chromatography system, always prior to measurement of each sample-batch.

2.4.3 Chemical element analysis

The chemical element measurements were done using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) in IACR-Rothamsted Research, Harpenden, UK and kindly provided by Dr. Malcolm J. Hawkesford.

2.4.4 Metabolome analysis by *Metanomics*

Metabolite pools in this study have been determined by *Metanomics* company (www.metanomics.de), which operates two highly complementary mass-spectrometry technologies, allowing the reliable monitoring of the wide range of chemical classes of metabolites.

2.4.5 Anthocyanin measurement

For anthocyanin extraction, 500 mg of frozen ground leaf material was used with 750 µl of 1 % (v/v) HCl in methanol. The homogenates were incubated for 24 h in 4° C. 500 µl of water and 700 µl of chloroform was added and after vigorous vortexing centrifuged for 3 min at 6000g at RT. 1 ml of the aqueous-methanol upper phase was collected for photometrical measurement: at 530 nm for anthocyanins and 657 nm for chlorophyll. For the chlorophyll content, the chlorophyll absorbance value was subtracted from the anthocyanin absorbance value. The results were obtained in relative values, always compared to these obtained for the wild type.

2.5 Bioinformatics tools and computer analysis

Arabidopsis sequence comparisons were performed using the BLAST (http://www.arabidopsis.org/Blast/) or WU-BLAST (http://www.arabidopsis.org/wublast/index2.jsp) programs with the standard parameters. For alignment of two sequences the BLAST2 program

(http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) was used with standard parameters. SALK *Arabidopsis* knock-out lines were identified using the T-DNA Express tool (http://signal.salk.edu/cgi-bin/tdnaexpress). Gene names were provided as the AGI codes following *AraCyc* nomenclature (TAIR; www.arabidopsis.org). Duplicated chromosome segments were identified using the final TIGR Arabidopsis genome annotation release version 5.0 (http://www.tigr.org/tdb/e2k1/ath1/Arabidopsis_genome_duplication.shtml).

Data calculations and visualization was performed using Excel, Word and Power Point (Microsoft Office 2003) programs. Photos were mostly prepared using Adobe™ Photoshop 7.0

2.5.1 Statistical data evaluation

All statistical analysis were carried out using Excel (Microsoft Office 2003). Significance of differences between means of data sets was determined using the student's t-test (heteroscedastic and double-sided). Differences between data sets were regarded as significant when probability of error was below 5 % ($P < 0.05$).

2.5.2 *MapMan*

The data obtained from ATH1 chip hybridisation were visualised using the *MapMan* software (Thimm et al., 2004; Usadel et al., 2005). A downloadable version for local application and a servlet version are available at http://gabi.rzpd.de/projects/MapMan/.

2.5.3 *Genevestigator*

Searching the public available arrays (ATH1 full genome arrays and Nottingham Arabidopsis Stock Centre Transcriptomics Service arrays) was done using the *Genevestigator* database (https://www.genevestigator.ethz.ch/), which comprises thousands of Arabidopsis arrays processed to date (Zimmermann et al., 2004). Responses of investigated TF genes to individual stresses were viewed by the *Response Viewer*. Ratios calculated from signal levels near background (<200) are considered to be noisy and often artificial, thus, were not analysed.

2.5.4 Biosystem response network reconstruction

Basic underlying dataset for the response network compiles S-starvation transcript and metabolic profiles data from the experiment presented in this work and experiments performed before (Nikiforova et al., 2003), and does not regard the replenishment studies. Network reconstruction was performed by Dr. Victoria Nikiforova, as described before (Nikiforova et al., 2005a).

3 RESULTS

Sulphate acquisition and assimilation in plants is regulated at many different levels, including the level of transcription. Regulation of gene transcription involves transcription factors (TFs). To identify TF genes that may be involved in S-regulation, we begun with the assumption, that such TF genes may be regulated by S-starvation and/or S-supply in plants. Two different technologies were used to identify such transcription factors: real time RT-PCR and Affymetrix arrays.

The first approach (3.A) presents the selection process of S-regulated TFs in the axenic cultures experiment (work done in collaboration with Dr Wolf Ruediger Scheible and Dr Rosa Morcuende) and preliminary results of functional characterization of selected transcription factors, using transgenic *Arabidopsis* knock out lines.

Section 3.B describes the functional characterization of two AP2 genes selected in the microarray hybridisation transcript profiling from 2003 (Nikiforova at al. 2003). The gain of function approach was taken. The transgenic over-expressing lines used in this approach were kindly provided by Dr Isabell Witt and Dr Maria Ines Zanor (then members of Plant Signaling Group, MPI-MP, Golm, Germany

3.A Identification of transcription factors involved in S-regulation in sulphur starvation and re-supply experiment

The real time reversed transcription (RT)-PCR-based platform for 1465 *Arabidopsis* transcription factors (TFs) (Czechowski et al., 2004; see also chapter 2.3.4.1) was used to identify putatively sulphur-regulated TFs. RNA was extracted from axenically grown plants and analyzed on Affymetrix ATH1 arrays and by quantitative RT-PCR to enable direct comparison between both datasets (Scheible et al., 2004). TF candidates genes obtained from that screening were tested by a reverse genetics approach, using *Arabidopsis* T-DNA knock out lines.

3.A.1 Experimental setup and physiological features of Arabidopsis seedlings grown in liquid cultures

Arabidopsis seedlings (first experimental set of plant material kindly provided by Dr Rosa Morcuende) were grown in liquid culture in 30 ml of sterile liquid full nutrition medium or 150µM SO_4 medium. Full nutrition medium provided the seedlings sufficient sulphate and was used as a control. The 150µM sulphate in the full nutrition medium allowed seedlings to germinate and grow, however, after 7 days of cultivation, the sulphate in that medium was consumed by plants and decreased to the

immeasurable amounts (data not shown). This forced plants to mobilise sulphate resources stored in their vacuoles. Transferring pre-grown 7-days seedlings to the S-depleted medium assured following S-starvation during next two days of plant cultivation.

Nevertheless, S-deprived seedlings exhibited no typical phenology of S-limited plants (data not shown), which are known to be: reduced chlorophyll, accumulation of anthocyanins in the leaves, and pronounced root and especially lateral root growth (Nikiforova at al., 2003). On day 9, all seedlings (+S and –S) had developed cotyledons and first leaves and remained green.

To minimise the unspecific influences of environmental factors, plant material from two simultaneously grown flask-cultures was combined for the same experimental point and, additionally, two independent experimental sets were carried out at an interval of around one year.

Eventually, eight different samples corresponding to four time points (full nutrition [FN], plants starved for 48h [-S], plants re-supplied with sulphate for 30 minutes [30 min S] and plants re-supplied with sulphate for 3 hours [3 h S]) from two independent experimental replicas (Experiment 1 and Experiment 2) were subjected to further analysis (see also chapter 2.2.3).

3.A.2 Expressional and metabolic responses to the S deprivation and re-addition as an induction control in both experimental replicas

Due to an absence of visible evidences for S-deprivation in experimental sets of seedlings, the occurrence of sulphate-depletion induced changes had to be proven in these plants. Therefore the S-starvation status in plants had to be documented to guarantee the further selection of a representative set for the sulphur-dependent TF candidates.

3.A.2.1 Content of thiols in S-starved and re-supplied plant material

As thiols represent the major sulphur components and immediate products of sulphate assimilation, determination of thiol levels was commonly used to reveal the metabolic responses of plants to changes of sulphate regimes.

In *Arabidopsis* seedlings, in two independent experiments, levels of all four thiol-compounds decreased significantly after 2 days of sulphur starvation, when comparing to plants grown on sulphur-sufficient medium. The reduced sulphate availability caused a block of cysteine synthesis as insufficient amounts of sulphide were provided through the uptake and sulphate reduction pathway. As expected, this led in a classical way to a reduction of the immediate products, cysteine, glutathione and the glutathione precursor – γ-glutamylcysteine (γ-Glu-Cys). Also the level of homocysteine, which is the precursor of methionine, was reduced. Under conditions of sulphur depletion, internal levels of cysteine decreased on average 3-fold (Fig. 3-1). Depending on the experiment, glutathione decreased 9,8 and 5 times, compared to control plants, γ-Glu-Cys 6,4 and 2,8 times and homocysteine 5,2 and 3,4 times, for experiment 1 and 2, respectively.

Re-addition of sulphate caused the reversed response of starved plants. Except for homocysteine, which levels did not change significantly even after 3 hours of sulphur replenishment,

cysteine, gluthatione and γ-Glu-Cys accumulated already after 12 minutes (in the 1st experiment) and 30 minutes (in the 2nd experiment) after SO_4^{2-} supply, compared to starved seedlings (Fig. 3-1). After 3 hours of SO_4^{2-} re-supply, cysteine increased on average 6-fold, gluthatione 8,7 and 5,5-fold and γ-Glu-Cys 6 and 3-fold, for experiment 1 and 2, respectively. The level of gluthatione and γ-Glu-Cys reached the initial status of plants grown under full nutrition, while cysteine exceeded this level up to 2-fold.

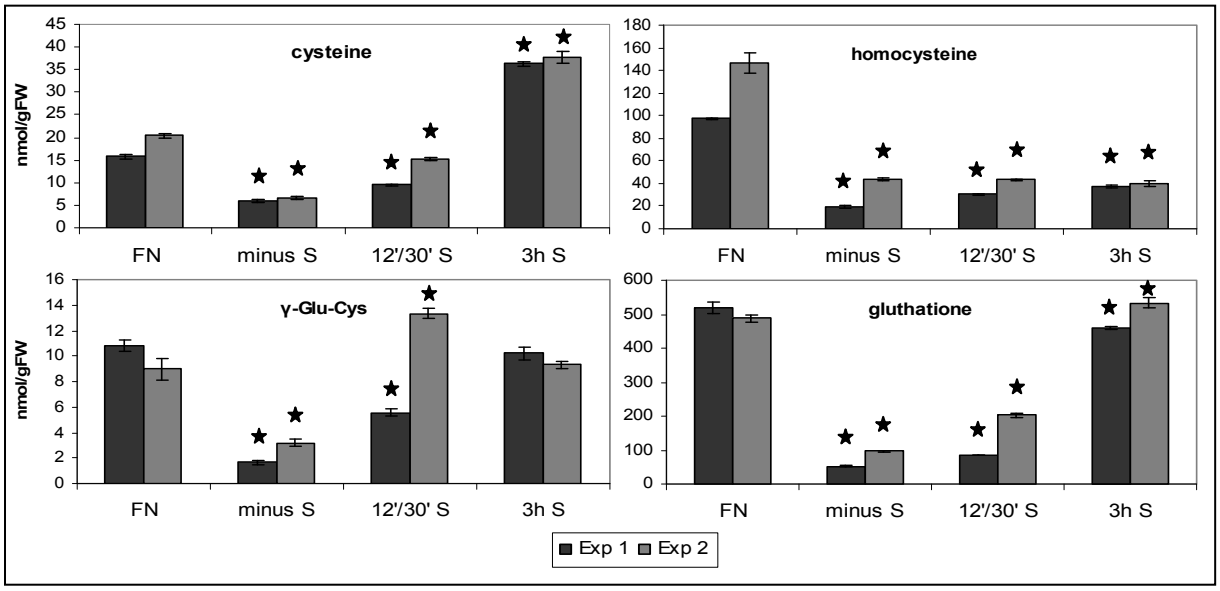


Fig 3-1 Levels of thiol-compounds in *Arabidopsis* seedlings grown in liquid cultures under different sulphur regimes, in two independent experiments. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different (P < 0,05) in comparison to the respective FN controls. (Exp, experiment; FN, full nutrition; S, re-supplied sulphate).

3.A.2.2 Sulphate and nitrate content

The content of anions like sulphate, nitrate and phosphate was determined as a further indicator for the nutrient status. Sulphate, nitrate and phosphate levels were measured using the ion chromatography. As expected, measurements revealed strong significant decreases (25 and 16-fold, for experiment 1 and 2, respectively) of sulphate after 2 days of sulphur starvation (zero sulphate medium) (Fig. 3-2). Starved seedlings contained only 4% and 6% (for experiment 1 and 2, respectively) of the sulphate level measured in control plants grown on full nutrition medium.

A slight accumulation of sulphate is detected only after 3h of sulphate re-addition, giving 3 and 1,5-fold higher sulphate compared to starved plants, which nevertheless is still 8 to 11 times less than in plants grown under full nutrition (Fig. 3-2).

Internal levels of nitrate differ between experiments in starved and replenished plant samples. In the 1st experiment this level is on average two times lower than in full nutrition or in starved and replenished samples from the 2nd experiment (Fig. 3-2). The only possible reason for that might be slightly lower amount of seeds used for each liquid culture prepared in 2nd experiment, which resulted in higher nitrate resources for growing plants. Nevertheless, despite the obvious differences, a nitrate concentration between 5 and 10mM/gFW is still insufficient to result in nitrate starvation of plants, which would have otherwise impaired the experiment (see also fig 3-5).

Phosphate levels remained constant and independent from the sulphate changes in both experiments performed (data not shown).

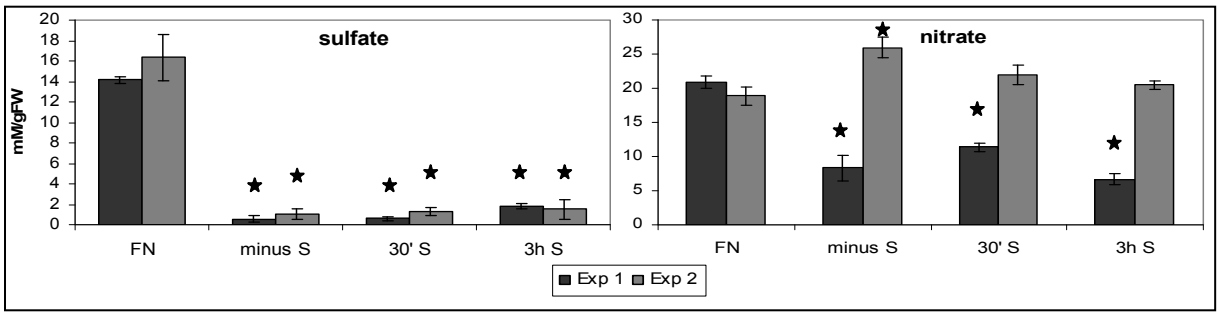


Fig 3-2 Levels of sulphate and nitrate determined by ion chromatography, in *Arabidopsis* seedling grown in liquid cultures under different sulphur regimes, in two independent experiments. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different ($P < 0,05$) in comparison to the respective FN controls. (Exp, experiment; FN, full nutrition; S, re-supplied sulphate).

3.A.2.3 Chemical element analysis

To confirm the metabolic status of the plants starving from sulphur deficiency, the total content of elemental sulphur was measured. The measurements were done using ICP-AES in IACR-Rothamsted Research, Harpenden, UK and kindly provided by Dr. Malcolm J. Hawkesford. The applied method allowed also to establish the levels of other chemical elements. Comparison of contents of all measured elements, in two independent experiments, is summarised in figure 3-3.

After 2 days of sulphur starvation (zero sulphate medium), plants pre-grown on medium containing 150µM sulphate contained only 21% and 22% (for experiment 1 and 2, respectively) of the internal total sulphur level measured in control plants grown on full nutrition medium (Fig. 3-3). A slight increase of total sulphur level is detected only after 3h of sulphate re-addition, giving 1,2-fold higher sulphur compared to starved plants (Fig. 3-3). The same changes were observed in both independently performed experiments.

The internal levels of most elements determined, like phosphorus (P), sodium (Na), potassium (K), ferrum (Fe) and zinc (Zn), remain constant and independent from the sulphate changes in both experiments performed (Fig 3-3). Levels of magnesium (Mg) and calcium (Ca) decreased slightly during experiment 2, being lower in starved and replenished seedlings, than in control (FN). In the 1st experiment, lower internal levels of Mg and Ca were detected only in the last experimental time point, after 3 hours after sulphate re-addition.

For some elements (Fe, Zn, Mg, Ca), small differences in their internal levels between two experimental sets were revealed. Levels of these elements are slightly elevated (for Fe, maximally 3 times higher) in the experiment 2, which again might be caused by slightly lower amount of seeds used for each liquid culture prepared in 2nd experiment, resulting in higher nutrients resources for growing plants.

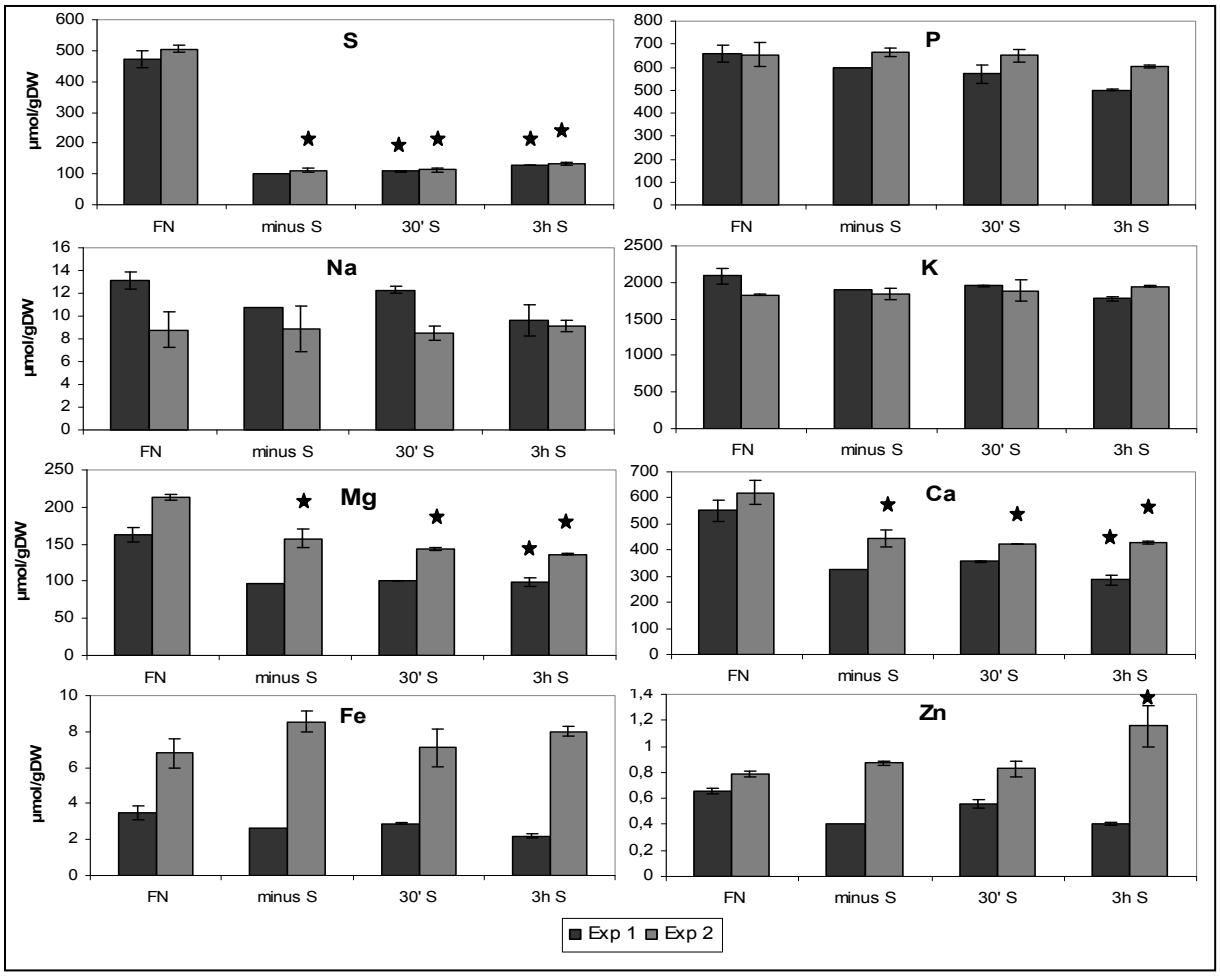


Fig 3-3 Levels of elements in *Arabidopsis* seedling grown in liquid cultures under different sulphur regimes, in two independent experiments. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different ($P < 0,05$) in comparison to the respective FN controls. (Exp, experiment; FN, full nutrition; S, re-supplied sulphate).

3.A.2.4 Metabolome analysis of *Arabidopsis* seedlings in response to sulphur deprivation

Metabolite pool sizes represent the integration of disturbed biosynthetic pathways, altered gene expression levels and altered enzyme abundancies and activities and are thus a good indicator for the response of the entire system.

The metabolite profile of sulphur deficiency and replenishment for both experimental replicas is catalogued in appendix A . Data are presented for all known metabolites, which were identified at least for one out of two experiments. Values represent the averages out of three independent measurements. The highest changes detected in metabolite levels are depicted in figure 3-4.

With a limited input of sulphur, levels of sulphur-containing metabolites such as cysteine and gluthatione decreased (chapter 3.A.2.1), while the precursor, serine, accumulated (Fig. 3-4). Serine increased 7,3 and 2,8-fold, in experiment 1 and 2, respectively. The elevated level of serine remained significantly higher than in full nutrition even after 3 hours of S replenishment. As serine is linked closely to glycine formation (Li et al., 2003; Bauwe and Kolukisaoglu, 2003), the concurrent

accumulation of glycine under S-depletion follows the expectations (Fig. 3-4). The changes of glycine levels reflect the levels of serine, being 7,7 and 3,3-fold higher than in full nutrition and remaining elevated even in S-replenished seedlings.

Despite the fact that cysteine itself serves as precursor of methionine through a transsulphuration reaction (Hesse et al., 2004a; Hesse and Hoefgen, 2003), methionine levels were not grossly affected by limited input of sulphur. Internal levels of methionine decreased 1,2 and 1,8-fold (for the experiment 1 and 2, respectively) in S-depleted seedlings and kept decreasing even after sulphate re-addition (Fig.3-4). Other aspartate family members such as homoserine, threonine and isoleucine did not display unique responses. Homoserine was detectable only in the 1st experiment and revealed an almost 2-fold decrease in S-starved seedlings, although the difference was not significant (Fig.3-4). Sulphate re-addition did not prevent the decrease of internal homoserine levels in the first time point measured, however a slight accumulation was detected after 3 hours. Levels of threonine were 1,5 and 1,1-fold higher in S depleted conditions, while isoleucine remained unchanged (Appendix A).

The pyruvate-derived amino acids, alanine and valine increased slightly in content (almost 2 times for both in –S conditions), leucine levels remained constant (Fig. 3-4; Appendix A).

Re-channelling of assimilated carbon affected the tryptophane level, which accumulated 6 (in 1st experiment) and 2 times (in 2nd experiment) under sulphur depletion (Fig.3-4). Other members of the phosphoenolopyruvate amino acids family, such as phenylalanine and shikimate were not affected. Tyrosine was detectable only in the 1st experiment, exhibiting a 1,5-fold increase in content under S deficient conditions (Appendix A).

While sulphate assimilation is impaired, nitrogen assimilation continues and the relative ratio of N to S is shifted towards an excess of N (Hesse et al., 2004a; Kopriva and Rennenberg, 2004). Plants accumulate excessive nitrogen in N-rich amino acids, asparagine and glutamine. Although asparagine was not determined in any of the experimental replicas, glutamine showed a consistent tendency for accumulation under S limited conditions, being 1,8 and 2,6-fold significantly increased in content (for the experiment 1 and 2, respectively), when compared full nutrition (Fig.3-4). The levels of glutamine were kept elevated even after 3h of re-supply with sulphate. A similar tendency for accumulation under S deficiency was observed for citruline, which was 1,4 and 2,5-fold higher in these conditions than in FN. Levels of arginine, proline and glutamic acid, other members of α-ketoglutarate amino acid family, presented only marginal alterations, which nevertheless were not consistent within both experimental replicas (Appendix A).

The highest accumulation was detected for putrescine, as the conversion to its downstream polyamine, spermidine, was presumably blocked due to reduced SAM availability (Nikiforova at al., 2006; Nikiforova et al. 2005b), which is a direct derivative of methionine. Putrescine levels were 21,7 and 14,4-fold (for experiment 1 and 2, respectively) higher upon S-starvation, compared to full nutrition (Fig.3-4). Putrescine content remained elevated even after re-supply with sulphate.

Extending this analysis to the precursors of the various amino acid branches, only marginal alterations among such organic acids as pyruvate, succinate, fumarate and malate, were detected. Ratios of their internal levels in starved plants versus ratios in those grown on full nutrition and SO_4^{2-}

re-supplied, oscillate between 1,4 and 0,4 (Appendix A). Sugars, glucose, fructose, sucrose and raffinose did not present a consistent response pattern to S-deficient conditions (Appendix A), being up to 4-fold increased (sucrose) in 1st experiment, while in the 2nd one this induction was not confirmed.

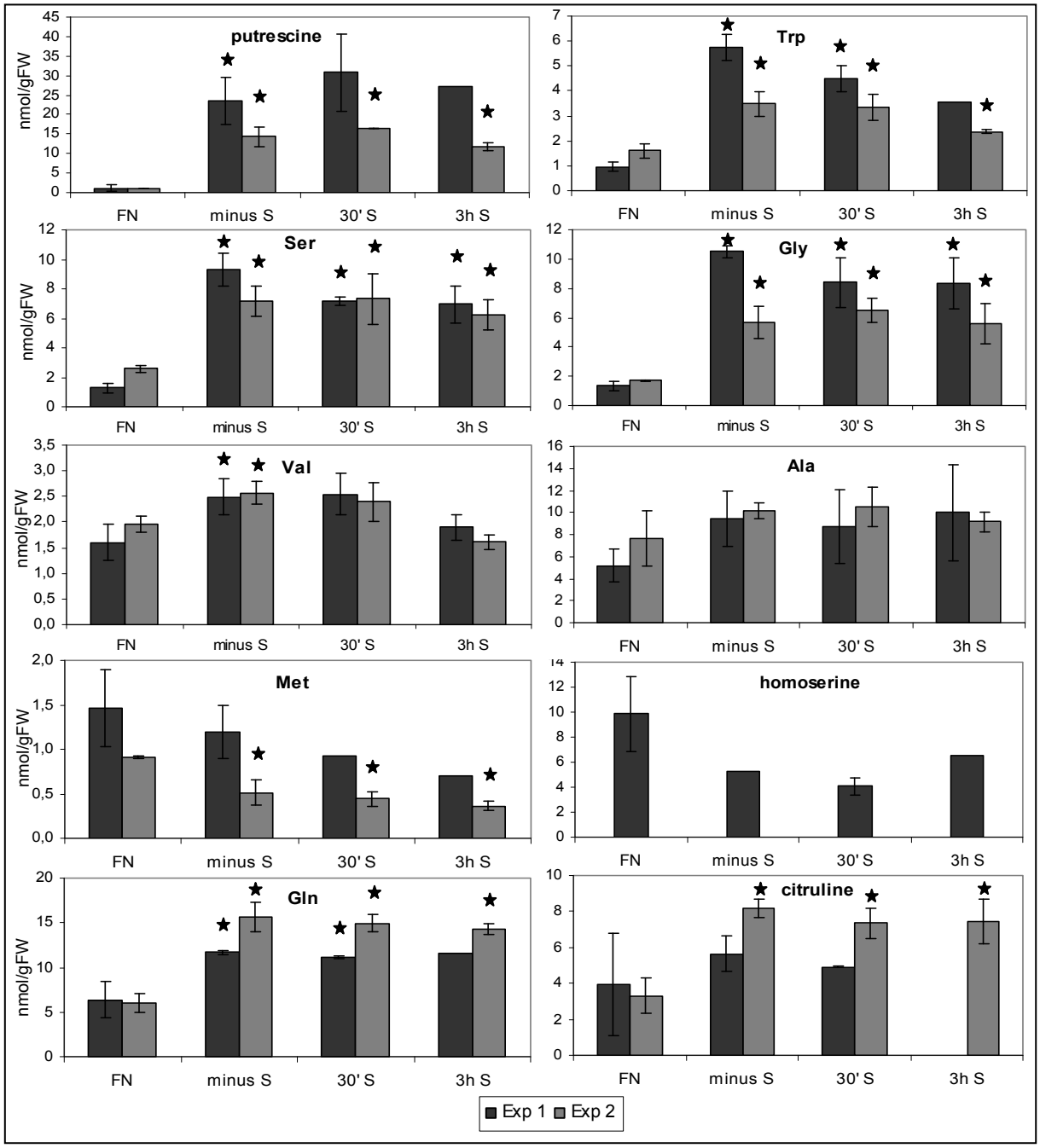


Fig 3-4 Levels of sulphur-related metabolites in *Arabidopsis* seedling grown in liquid cultures under different sulphur regimes in two independent experiments. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different ($P < 0,05$) in comparison to the respective FN controls. (Exp, experiment; FN, full nutrition; S, re-supplied sulphate)

Interestingly, two metabolites from vitamin E biosynthesis pathway were changed under S limited conditions. γ-tocopherol, a direct precursor of α-tocopherol (the major vitamin E compound found in leaf chloroplasts), and its precursor, 2,3-dimethyl-5-phytylquinol, were both elevated in

content 2,35 times under –S conditions (in the 2nd experiment), when compared to full nutrition conditions (Appendix A; Munne-Bosh, 2005). After sulphate re-supply, their levels decreased 0,6-fold, compared to starved plants, however, did not reach the full nutrition levels even after 3 hours of re-supplementation. Unfortunately, the measurements from the 1st experiment gave for these metabolites statistically non-reliable results (Appendix A). Tocopherols are derivatives of tyrosine, which levels were unchanged in our experimental conditions. Interestingly, in subsequent reactions the S-adenosylmethionine (SAM) serves as a methyl donor for the synthesis of 2,3-dimethyl-5-phytylquinol, α-tocopherol and β-tocopherol. Tocopherols are presumed to be important antioxidants deactivating photosynthesis-derived reactive oxygen species and preventing the propagation of lipid peroxidation by scavenging lipid peroxyl radicals in thylakoid membranes. Changes in α-tocopherol levels result from altered expression of pathway-related genes, degradation and recycling, and it is generally assumed that increases of α-tocopherol contribute to plant stress tolerance, while decreased levels favor oxidative damage. Recent studies indicate that the whole set of antioxidant defenses (such as: ascorbate, glutathione, carotenoids, tocopherols and other isoprenoids, flavonoids and enzymatic antioxidants) rather than a single antioxidant exists to afford adequate protection to the photosynthetic apparatus and helps plants to withstand environmental stress (Munne-Bosh, 2005; Hollaender-Czytko et al, 2005).

Metabolite measurements allowed to establish the Gln/Glu ratio, which can be used as an indicator for S-starvation effect on N metabolism. The elevated Gln/Glu ratio, observed in both experimental replicas, is characteristic for S-starvation (Fig 3-5). Decreased Gln/Glu ratio is typically characteristic for N-starvation, which is not the case in our experimental conditions (see chapter 3.A.2.2). In experiment 2 this ratio is slightly lower than in the 1st one, indicating less stronger induction followed by S-depletion, which is confirming the same tendency visible in all data obtained for thiols, ions, elements and metabolites content in plants.

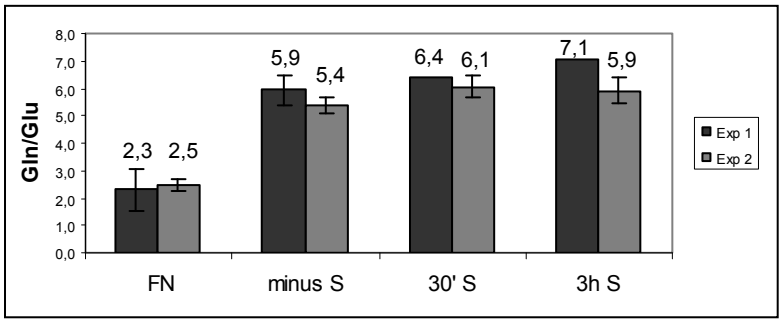


Fig 3-5 Relative ratio of Gln to Glu in *Arabidopsis* seedlings grown in liquid cultures under different sulphur regimes, in two independent experiments.

3.A.2.5 Testing the primary S-assimilation genes

When the capacities for pool size regulation by the existing enzyme machinery are exhausted due to depletion of affected precursors pools, the plant is forced into a response cascade resulting in an adjustment of the enzyme composition fitting to the altered environmental conditions. After sensing disbalances in nutrients or metabolites, alterations in gene expressions are triggered. Thus, prior to

Table 3-1 Relative expression level of genes involved in primary sulphate assimilation pathway. Ratios higher than 2,0 are depicted in bold, red – induced, black – repressed. Genes, in which promoters region (-3-kb upstream sequence) the SURE core sequence was found (Maruyama-Nakashita et al., 2005), are marked with (1).

S-PATHWAY GENES		1st experimental set						2nd experimental set					
		Q-RTPCR			AFFYMETRIX			Q-RTPCR			AFFYMETRIX		
AGI code	Gene name	-S vsFN	30' vs-S	3h vs-S	-S vsFN	30' vs-S	3h vs-S	-S vsFN	30' vs-S	3h vs-S	-S vsFN	30' vs-S	3h vs-S
At4g08620[1]	Sultr1;1	**26,893**	0,822	**0,167**	**24,673**	1,246	**0,353**	**26,265**	1,726	**0,236**	**29,304**	0,933	**0,342**
At1g78000	Sultr1;2	**18,652**	0,625	**0,116**	**16,225**	0,686	**0,210**	**14,199**	1,135	**0,182**	**16,586**	0,535	**0,209**
At1g22150	Sultr1;3				**14,750**	**0,314**	1,102	**0,102**	1,778	**4,685**	1,600	2,000	1,063
At5g10180[1]	Sultr2;1	**6,532**	0,743	**0,156**	**5,377**	0,811	**0,283**	**6,134**	1,187	**0,201**	**2,639**	0,916	**0,348**
At1g77990	Sultr2;2				**4,202**	0,945	**0,318**	**4,820**	1,155	**0,313**	**12,027**	0,730	**0,339**
At3g51895	Sultr3;1				1,112	0,897	1,061	1,478	1,226	1,085	0,929	1,288	1,110
At4g02700	Sultr3;2				0,979	0,975	0,905	0,627	1,889	1,277	0,917	0,974	1,056
At1g23090	Sultr3;3				0,944	1,304	1,481	unspecific product			0,652	1,112	1,389
At3g15990	Sultr3;4				**5,010**	1,562	0,647	**3,210**	1,991	0,841	0,898	1,687	1,276
At5g19600	Sultr3;5				0,736	0,677	1,631	**0,278**	1,069	**4,994**	1,039	1,347	1,532
At5g13550	Sultr4;1				**6,590**	0,689	**0,262**	**5,035**	1,168	**0,376**	**3,527**	0,747	**0,307**
At3g12520[1]	Sultr4;2				**21,761**	0,793	**0,119**	**15,053**	1,225	**0,157**	**10,862**	0,754	**0,164**
At1g80310	Sultr5;1				0,877	1,140	1,392	0,662	1,798	**2,031**	0,660	1,326	1,220
At3g22890	ATPS1				0,857	0,617	0,676	0,935	0,880	0,721	0,735	0,745	0,778
At1g19920	ATPS2				**0,268**	1,269	**3,212**	**0,188**	2,332	**3,863**	**0,273**	1,187	**3,760**
At4g14680	ATPS3				**3,713**	**0,377**	**0,281**	**4,020**	**0,348**	**0,243**	**2,892**	**0,426**	**0,382**
At5g43780	ATPS4				**0,134**	0,977	0,897	**0,156**	1,321	0,774	**0,143**	0,999	0,801
At2g14750	APSK1				1,298	0,995	0,949	1,183	1,315	1,045	0,989	0,905	1,369
At4g39940	APSK2				**0,356**	1,452	**3,077**	**0,332**	2,274	**4,089**	**0,407**	1,278	**3,460**
At4g04610	APR1	**7,979**	0,247	0,233	**5,895**	**0,305**	**0,284**	**7,678**	0,528	**0,317**	**4,942**	**0,267**	**0,314**
At1g62180	APR2	**3,230**	0,249	0,198	**3,799**	**0,211**	**0,214**	**3,169**	**0,490**	**0,257**	**4,495**	**0,206**	**0,232**
At4g21990[1]	APR3	**33,313**	0,188	0,042	**29,042**	**0,353**	**0,061**	**24,997**	**0,419**	**0,060**	**10,791**	**0,250**	**0,101**
At5g04590	SulphitRed				1,222	0,829	1,284	unspecific product			1,262	0,871	1,065
At4g35640	SAT				not present			**34,939**	0,689	**0,086**	not present		
At1g55920	SAT 1	2,027	0,869	0,569	**2,921**	1,021	0,704	**1,931**	1,422	0,816	**2,059**	0,812	0,784
At3g13110	SAT A	1,353	1,070	0,724	1,907	0,991	0,812	1,419	1,571	1,015	1,163	1,055	1,050
At5g56760	SAT 52				1,387	1,028	1,061	1,077	1,348	1,140	1,241	1,005	0,954
At2g17640	SAT2 106				1,791	**0,429**	0,544	**5,485**	0,751	**0,241**	**2,488**	0,522	0,510
At4g14880	1OASTLA1				0,955	0,786	0,792	0,608	1,217	0,947	0,975	1,047	0,930
At2g43750	2OASTLB				1,508	0,682	0,620	1,118	1,311	0,714	1,991	0,938	0,648
At3g59760	3OASTLC				1,102	0,929	1,366	undetectable			0,830	0,964	1,386
At3g03630	4OASTL26				**0,098**	**0,194**	**2,538**	undetectable			0,646	0,716	**2,068**
At3g04940	5OASTLD1				0,616	1,138	1,016	0,507	1,938	1,352	0,918	0,945	1,170
At3g22460	6OASTL				1,348	0,892	0,641	0,821	1,983	0,874	1,201	0,945	0,685
At3g61440	7OASTLC1				0,826	1,030	1,158	0,641	1,566	1,390	0,922	0,989	1,221
At5g28020	8OASTLD2				1,465	0,914	0,548	undetectable			**2,126**	0,968	0,642
At5g55880	10OASTLn				not present			undetectable			not present		

the entire TF-library screening by quantitative RT-PCR, qRT-PCR analysis for several genes involved in primary assimilation of sulphate, were performed (Nikiforova et al., 2003; Hirai and Saito, 2004; for primer sequences see appendix E).

As controls for S-starvation status two high affinity sulphate transporter genes SULTR 1;1, SULTR 1;2, one low affinity sulphate transporter SULTR 2;1, and all three isoformes of APS reductase were analysed. All of those genes show very consistent induction patterns in both experimental replicas, being strongly induced after a starvation period of 48 hours (Table 3-1). Moreover, after adding back sulphate to the cultivation medium, induction of these genes was in most cases repressed, which manifested in ratios (replenished samples versus starved samples) lower than 1. The same result was achieved when using quantitative RT-PCR or Affymetrix chip hybridisation methods. Table 3-1 presents the relative expression levels from qRT-PCR compiled together with the data from the Affymetrix chips hybridisation for both experimental replicas (see also chapter 3.A.3).

3.A.3 Global expression profiling by DNA macroarray (Affymetrix chip)

As a part of a wider screen to identify candidate genes regulated by changes in sulphate availability we performed expression profiling using Affymetrix ATH1 array. Transcript levels of 22750 genes for two independent experiments (see chapter 2.2.3) were estimated, resulting in eight datasets coming from two biological replicates (four time points each). Depending on the experiment 28% to 22% of the genes were called 'absent' by Affymetrix microarray suite software (MAS version 5.0), being not detectable in any experimental point in the first or second experiment, respectively.

The changes between transcript levels under different sulphate regimes were analysed by calculating the ratio (R) of gene expression levels in full nutrition or after SO_4^{2-} addition relative to the level in S-starved seedlings. For all genes, R more than 5 or less than 0,2 at least in one of three comparisons was the criteria used to identify genes responding significantly to the experimental conditions of sulphur depletion or re-supply, which were termed as sulphur-responsive genes.

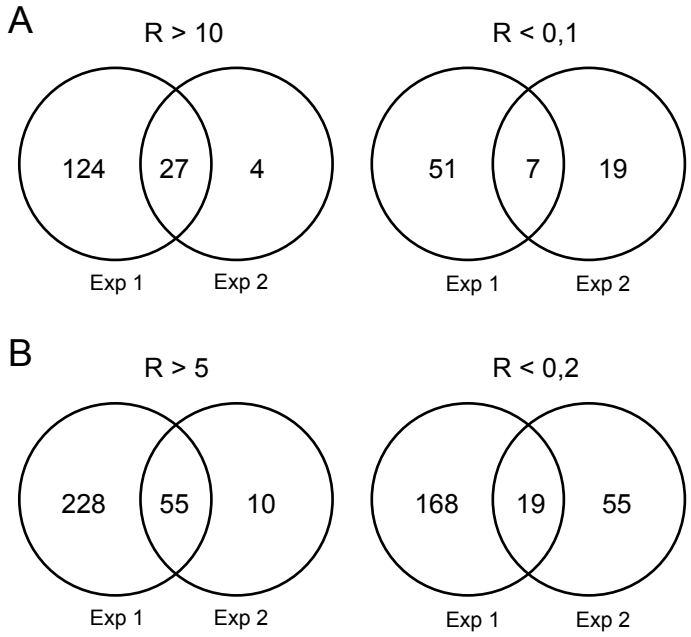


Fig 3-6 Comparative analysis of the gene expression patterns under sulphur deficiency stress in two independent experimental replicas. Venn diagrams show the numbers of genes more than 10-fold **(A)** and more than 5-fold **(B)** significantly up- and downregulated in at least one experiment; R, ratio of Affymetrix chips signal intensity at –S to signal intensity in full nutrition.

Reproducibility between the two biological replicates was assessed by comparing ratios (–S versus FN) for genes assigned as 'present' by MAS software in S-deprived samples or full nutrition samples for up-regulated or down-regulated genes, respectively. In the first experiment 384 genes yielded gene expression ratios higher than 5 and lower than 0,2; 283 up-regulated genes and 65 genes exhibited reduced expression. The second experiment yielded less genes which exceeded the fixed threshold. In total, 261 genes changed their transcript abundance significantly, among them 187 genes were over-regulated in S-deficient conditions and 74 were down-regulated. The number of genes showing a significantly altered expression level under sulphur deficiency is rather low and was the main difference between two independent experimental replicates. The number of S-responsive genes overlapping between both experiments is also quite low: 55 up-regulated and 19 down-regulated genes (Fig 3-6; Table 3-2). When comparing genes which exceeded the threshold by level of 10, percentage of overlapping genes does not change and oscillate around 19% (1st Exp) and 86% (2nd Exp) for overexpressed genes and around 11% (1st Exp) and 26% (2nd Exp) for down-regulated ones. However, of 124 non-overlapping, 10 times overexpressed genes from the 1st experiment, 20 exhibited 5 fold overexpression in the 2nd experiment and locate among 65 overexpressed genes. Thus the low level of overlapping genes arises from different strength of S-induction in both experiments rather than from other influences resulting in selection of various, non-overlapping set of genes. The 2nd experiment revealed to be less severe in terms of S-starvation than the 1st one. Appendix B comprises 233 genes which exceeded the threshold by level of 10 in at least one experimental replica.

Functional analysis was achieved by grouping sulphur deficiency-responsive genes according to the predicted functions of their proteins. The classification uses groups defined in *MapMan* software (Thimm et al., 2004; Usadel et al. 2005). Table 3-3 shows the proportion of genes in different functional classes that exhibited S-dependent changes in expression. In 17 of the 30 functional categories, the number of genes up-regulated at –S was higher than the number of down-regulated genes. This disproportion is especially high in transport category, but also in lipid metabolism and the protein posttranslational modification group. The opposite situation revealed the protein synthesis and targeting group, comprising exclusively down-regulated genes. S-responsive changes are also frequent in regulation of transcription, secondary metabolism, hormone metabolism and enzyme class, more equally contributed by up- and down-regulated genes.

As already described in section 3.A.2.1 the profiles of –S regulated genes indicated recruitment of genes for sulphate uptake and assimilation (Table 3-1). It is suggested that induced expression of high affinity SULTR1;1 (*At4g08620*) and SULTR1;2 (*At1g78000*) enhances the sulphate uptake capacity of root epidermis cells (Takahashi et al., 2000). Low affinity SULTR2;1 (*At5g10180*), expressed in vascular tissues is also known to be strongly induced by sulphur starvation. Two sulphate transporters predicted to be localised in chloroplast membrane, SULTR4;1 (*At5g13550*) and SULTR4;2 (*At3g12520*), also revealed elevated transcripts abundancies in tested conditions. Up-regulation of all three isoforms of APS reductase (*At4g04610*, *At1g62180*, *At4g21990*), suggests that activation of this key metabolic pathway facilitates efficient synthesis of cysteine under S-deprived conditions.

Table 3-2 Relative expression level of 74 sulphur responsive genes, which were up- or down-regulated more than 5 times (5<R<0,2) under S-deficient conditions in both experimental replicas. Subset of 34 genes, which expression was changed more than 10 times (10<R<0,1) under sulphur starvation, is depicted in bold.

		1st Experiment			2nd Experiment				
Gene ID	**Affymetrix spot ID**	**S starv vs FN**	**30' vs S starv**	**3h vs S starv**	**S starv vs FN**	**30' vs S starv**	**3h vs S starv**	**Gene description**	**Functional category**
At5g23010	249866_at	0,033	0,591	11,500	0,044	1,243	15,521	2-isopropylmalate synthase-like	amino acid metabolism.Ala-Val-Leu.synth
At3g19710	257021_at	0,018	1,097	14,578	0,025	1,078	19,163	branched-chain aa aminotransferase, put	aa metabolism.branched chain aminotransferases
At3g08860	258983_at	153,064	0,753	0,053	36,791	0,699	0,063	putative aminotransferase	amino acid metabolism.alanine.synthesis
At4g34710	253203_at	31,131	0,932	0,310	15,130	0,878	0,344	arginine decarboxylase SPE2	amino acid metabolism.arginine.degradation
At4g23990	254185_at	9,807	0,874	0,273	6,252	0,891	0,330	AtCslG1 cellulose synthase - like protein	cell wall.cellulose synthesis
At2g23060	267250_at	29,308	0,685	0,459	8,806	0,458	0,220	similar to hookless1 (HLS1)	development.unspecified
At1g34310	262566_at	0,073	3,715	3,567	0,190	1,581	2,728	auxin response factor 1, putative	hormone metabolism.auxin.signal transduction
At1g05680	263231_at	28,979	0,702	1,509	5,418	1,328	4,267	indole-3-acetate beta-glucosyltransferase, put	hormone metabolism.auxin.synthesis-degradation
At5g48180	248713_at	16,720	0,762	0,204	5,777	0,854	0,227	putative protein	hormone metabolism.jasmonate regulation
At1g76690	259875_s_at	12,064	0,729	0,307	6,025	0,806	0,476	12-oxophytodienoate reductase (OPR2)	hormone metabolism.jasmonate.synthesis-degrad
At1g74460	260234_at	12,211	0,901	1,041	5,107	1,216	1,167	putative lipase/acylhydrolase	lipid metabolism.lipid degradation
At1g19610	261135_at	0,063	0,396	0,302	0,190	1,031	0,802	defensin AMP1, putative	metal handling.binding, chelation and storage
At3g28740	256589_at	148,909	0,518	0,825	11,325	0,878	1,276	cytochrome P450, putative	misc.cytochrome P450
At5g23190	249881_at	19,520	0,880	0,782	7,242	1,254	0,946	cytochrome P450-like protein	misc.cytochrome P450
At1g16410	262717_s_at	0,064	0,393	9,574	0,050	1,409	15,364	putative cytochrome P450	misc.cytochrome P450
At2g22330	264052_at	0,065	1,218	13,502	0,162	1,428	11,907	putative cytochrome P450	misc.cytochrome P450
At4g13770	254687_at	0,025	1,052	9,701	0,061	1,201	8,801	cytochrome P450 monooxygenase (CYP83A1)	misc.cytochrome P450
At4g39950	252827_at	0,174	1,593	9,280	0,189	1,723	7,073	cytochrome P450 - like protein	misc.cytochrome P450
At2g44460	267389_at	363,511	0,746	0,026	551,894	0,769	0,010	putative beta-glucosidase	misc.gluco-, galacto- and mannosidases
At3g60140	251428_at	527,411	0,736	0,026	341,441	0,710	0,011	beta-glucosidase-like protein	misc.gluco-, galacto- and mannosidases
At1g78370	260745_at	0,147	0,903	6,921	0,175	1,030	8,046	2,4-D inducible glutathione S-transferase, put	misc.glutathione S transferases
At3g22740	258322_at	0,093	0,245	0,633	0,087	1,061	3,023	putative selenocysteine methyltransferase	misc.misc2
At3g44320	252677_at	5,697	0,971	0,305	5,110	0,667	0,292	nitrilase 3	misc.nitrilases
At4g20820	254430_at	11,000	0,703	0,079	17,710	0,444	0,172	reticuline oxidase - like protein	misc.nitrilases
At1g75280	256454_at	6,551	0,803	0,330	8,014	1,010	0,300	NADPH oxidoreductase, putative	misc.oxidases - copper, flavone etc.
At1g75290	256450_at	90,500	0,537	0,085	18,467	0,677	0,076	NADPH oxidoreductase, putative s	misc.oxidases - copper, flavone etc.

At5g37980	249601_at	12,960	0,812	0,270	8,215	0,656	0,223	quinone oxidoreductase -like protein	misc.oxidases - copper, flavone etc.
At1g65860	261913_at	0,030	3,176	18,882	0,117	0,879	6,780	flavin-containing monooxygenase FMO3, put	misc.oxidases - copper, flavone etc.
At2g15490	265501_at	40,600	1,064	0,926	9,600	1,174	1,799	putative glucosyltransferase	misc.UDP glucosyl and glucoronyl transferases
At4g21990	254343_at	29,042	0,353	0,061	10,791	0,250	0,101	PRH26 protein	not assigned.no ontology
At5g09520	250541_at	15,408	0,677	0,545	6,583	1,213	0,964	putative proline-rich protein	not assigned.no ontology
At5g09530	250500_at	18,629	0,966	0,991	7,267	1,062	1,017	periaxin - like protein	not assigned.no ontology
At1g03700	264842_at	14,409	0,595	0,707	10,878	0,883	0,904	hypothetical protein	not assigned.unknown
At1g04770	261177_at	17,548	0,176	0,079	9,525	0,186	0,107	hypothetical protein	not assigned.unknown
At1g12030	257421_at	65,960	0,185	0,162	123,203	0,143	0,071	hypothetical protein	not assigned.unknown
At1g78990	257428_at	97,615	1,156	0,775	6,963	1,283	1,346	hypothetical protein	not assigned.unknown
At2g43390	260535_at	85,167	0,840	0,636	12,727	1,246	0,979	hypothetical protein	not assigned.unknown
At3g27150	256750_at	12,103	0,625	0,353	5,728	0,819	0,322	unknown protein	not assigned.unknown
At3g49580	252269_at	427,538	0,466	0,082	105,830	0,379	0,116	putative protein	not assigned.unknown
At4g01870	255543_at	52,586	0,812	1,385	5,260	1,682	3,496	hypothetical protein	not assigned.unknown
At4g20390	254474_at	7,505	0,967	0,965	5,931	1,213	1,687	putative protein	not assigned.unknown
At4g31330	253525_at	35,741	0,613	0,243	21,148	0,494	0,208	predicted protein	not assigned.unknown
At4g33960	253317_at	9,401	0,482	0,197	7,767	0,481	0,247	putative protein	not assigned.unknown
At4g38080	253024_at	10,090	1,021	0,827	5,349	1,148	1,320	putative protein	not assigned.unknown
At5g13900	250230_at	8,605	0,841	0,640	5,487	1,266	0,682	putative protein	not assigned.unknown
At5g24660	249752_at	46,719	0,331	0,132	21,925	0,343	0,098	putative protein	not assigned.unknown
At5g48850	248676_at	818,532	0,403	0,067	161,772	0,291	0,054	putative protein	not assigned.unknown
At1g47400	261684_at	0,147	0,753	0,765	0,104	0,644	0,732	hypothetical protein	not assigned.unknown
At2g32160	265698_at	0,174	3,606	1,091	0,159	0,120	1,160	hypothetical protein	not assigned.unknown
At3g05770	258739_s_at	0,005	3,000	3,000	0,026	13,600	15,000	unknown protein	not assigned.unknown
At5g05250	250828_at	0,186	1,552	1,322	0,145	1,155	1,110	unknown protein	not assigned.unknown
At1g73290	260091_at	33,667	0,990	1,000	9,556	1,035	0,721	putative serine carboxypeptidase	protein.degradation
At1g36370	260126_at	228,091	0,824	0,056	19,849	0,369	0,062	putative hydroxymethyltransferase	PS.photorepiration
At1g03020	263168_at	24,750	0,886	0,170	14,694	0,499	0,132	putative glutaredoxin	redox.glutaredoxins
At4g15690	245505_at	0,005	0,500	7,000	0,025	4,111	29,444	glutaredoxin	redox.glutaredoxins
At1g17950	255903_at	5,850	0,910	0,714	7,600	1,368	0,921	myb-like protein, putative	RNA.regulation of transcription
At5g43780	249112_at	0,134	0,977	0,897	0,143	0,999	0,801	ATP sulphurylase precursor	S-assimilation
At1g56650	245628_at	65,397	1,322	0,420	18,765	1,054	0,457	anthocyanin2, putative	secondary metabolism.flavonoids

At1g18870	261428_at	68,290	0,509	0,073	22,405	0,865	0,155	isochorismate synthase, putative	secondary metabolism.phenylpropanoids
At5g07130	250674_at	8,777	0,781	0,749	5,005	1,127	0,806	laccase-like protein	secondary metabolism.phenylpropanoids
At1g51830	246375_at	20,333	1,639	0,057	8,714	1,934	1,230	light repressible rec protein kinase, put	signalling.receptor kinases
At1g23730	265170_at	90,508	0,887	0,130	62,435	0,874	0,095	putative carbonic anhydrase	TCA / org. transformation.carbonic anhydrases
At2g43100	266395_at	0,178	0,797	6,072	0,177	1,021	6,192	3-isopropylmalate dehydratase	TCA / org. transformation.TCA
At3g58990	251524_at	0,114	1,349	14,232	0,134	1,009	11,225	3-isopropylmalate dehydratase-like prot	TCA / org. transformation.TCA
At1g66760	256324_at	24,057	1,221	0,126	13,185	0,865	0,164	MATE efflux family protein, putative	transport misc
At3g55090	251824_at	12,571	0,564	0,580	6,100	1,303	0,869	ABC transporter - like protein	transport.ABC transporters and multidrug resistance
At5g13580	250239_at	21,072	1,108	0,696	5,806	1,232	0,894	ABC transporter-like protein	transport.ABC transporters and multidrug resistance
At3g56200	251722_at	12,324	0,597	0,190	7,940	0,497	0,128	putative glutamine transporter	transport.amino acids and peptides
At5g18290	250025_at	11,615	0,525	0,145	11,377	0,563	0,125	putative protein	transport.Major Intrinsic Proteins.SIP
At1g78000	262133_at	16,225	0,686	0,210	16,586	0,535	0,209	high affinity sulphate transporter, put	transport.sulphate
At3g12520	256244_at	21,761	0,793	0,119	10,862	0,754	0,164	sulphate transporter, putative	transport.sulphate
At4g08620	255105_at	24,673	1,246	0,353	29,304	0,933	0,342	putative sulphate transporter	transport.sulphate
At5g26220	246884_at	107,658	0,420	0,035	184,330	0,367	0,023	cation transport protein chaC, E. coli, put	transport.unspecified cations
At3g05400	259133_at	113,586	1,095	0,062	28,894	0,783	0,060	sugar transporter, putative	transporter.sugars

Table 3-3 Distribution of differentially expressed genes between the groups of up- and downregulated in the functional categories at sulphur deficient conditions; R, ratio of signal intensity at –S to intensity in full nutrition; 0, no hit.

Category	Upregulated R>5	Downregulated R<0,2	Together 5<R<0,2
Amino acid metabolism	4	5	9
Cell cycle / division / organisation	2	3	5
Cell wall synthesis and modification	5	2	7
Co-factor and vitamine metabolism	1	0	1
CHO metabolism	4	3	7
Development	5	1	6
Fermentation	1	0	1
Glycolysis	1	1	2
Hormone metabolism	9	7	16
Lipid metabolism	13	2	15
Metal handling / chelation / storage	1	3	4
Miscellaneous / enzyme families	39	16	55
Mitochondrial electron transport / ATP synthesis	3	1	4
Not assigned no ontology/unknown	117	114	231
Nucleotide metabolism	0	2	2
OPP / oxidative PP	0	2	2
Protein synthesis / targeting	0	18	18
Protein degradation	5	2	7
Protein postranslational modification	6	1	7
PS lightreaction / calvin cycle / photorespiration	1	4	5
Redox	3	10	13
Regulation of transcription	14	10	24
Secondary metabolism	14	5	19
Signalling	8	5	13
S-assimilation	1	1	2
Stress biotic	6	7	13
Stress abiotic	5	4	9
TCA cycle	1	3	4
Tetrapyrrole synthesis	0	2	2
Transport	31	1	32

We also analysed our expressional dataset for reliability by comparison with results on sulphur starvation experiments made earlier by a number of research groups, where the behaviour of individual S-responsive genes was described (Nikforova et al., 2003; Maruyama-Nakashita et al., 2003; Hirai et al., 2003; Hirai and Saito, 2004). The transcript analysis revealed strong induction of genes related to the oxidative stress response such as two isoforms of putative isoflavonoid reductases (IFR; *At1g75280* and *At1g75280*) (Table 3-4). The exact function of this genes is yet to be characterised, though it is suggested to play a role in preservation of reductants or synthesis of antioxidants. In addition, we found significant overexpression of carbonic anhydrase (*At1g23730*), which may have a specific function to alleviate oxidative stress by fueling the respiratory cycle. Synthesis of jasmonate was up-regulated corollary of oxidative stress triggered by shortage of gluthation (GSH). Under limited sulphate conditions, it was manifested in elevated transcript

abundancies of genes encoding two JA biosynthesis enzymes such as 12-oxophytpdieonoate (OPR2; *At1g76690*) and lipoxygenase (AtLOX2; *At3g45140*). Although in our experimental conditions the accumulation of anthocyanins was not observed in S-deprived plants, genes suggested to be involved in the activation of flavonoid biosynthesis such as PAP1 transcription factor (*At1g56650*) were highly induced in these conditions (see chapter 3.A.4). The nitrilase 3 (*At3g44320*) induction under –S suggest enhanced auxin production or glucosinolate degradation, especially via conversion of indole-acetonitrile, the product of indole glucosinolates degradation (see below). Three of four isoforms of the protein of unknown function (*At3g49580*, *At5g24660*, *At5g24655*) were found to be induced under S-limited conditions, while after S re-supply their response was attenuated or even reversed. Moreover, for most of the S-responsive genes found in our experiment, the presence of conserved *cis*-acting element named SURE in their upstream regions, was demonstrated (Table 3-4; Maruyama-Nakashita et al., 2005).

Table 3-4 Relative expression levels of sulphur responsive genes. Genes significantly (present calls) up- and downexpressed (10<R<0,1) under sulphur starvation are depicted in bold. Genes already published as S-responding (Hirai and Saito;2004) are marked with (1). Genes, in which promoters region (-3-kb upstream sequence) the SURE core sequence was found (Maruyama-Nakashita et al., 2005), are marked with (2).

Gene ID	Gene name	1st Experiment			2nd Experiment		
		-S vs FN	30'S vs -S	3hS vs -S	-S vs FN	30'S vs -S	3hS vs -S
At3g49580[2]	unknown protein	427,54	0,47	0,08	105,83	0,38	0,12
At5g24660[1,2]	unknown protein	46,72	0,33	0,13	21,93	0,34	0,10
At5g24655	unknown protein	4,72	0,56	0,43	1,95	0,74	0,54
At1g75280[1]	isoflavone reductase (IFR), putative	6,55	0,80	0,33	8,01	1,01	0,30
At1g75290[2]	isoflavone reductase (IFR), putative	90,50	0,54	0,08	18,47	0,68	0,08
At3g05400[1]	sugar transporter, putative	113,59	1,09	0,06	28,89	0,78	0,06
At5g26220[1,2]	ChaC-like protein family of cation transporters	107,66	0,42	0,03	184,33	0,37	0,02
At1g36370[1, 2]	serine hydroxymethyltransferase/threonine aldolase, put	228,09	0,82	0,06	19,85	0,37	0,06
At3g44320[1]	nitrilase 3	5,70	0,97	0,30	5,11	0,67	0,29
At1g18870[1,2]	isochorismate synthase (isochorismate mutase), put.	68,29	0,51	0,07	22,41	0,87	0,16
At1g76690[1]	12-oxophytodienoate reductase (OPR2)	12,06	0,73	0,31	6,02	0,81	0,48
At1g75270[1]	dehydroascorbate reductase (DHAR), putative	4,07	0,82	0,55	3,71	0,75	0,53
At1g08830[1]	superoxide dysmutase (CSD1)	1,01	1,08	0,92	1,04	0,95	1,00
At2g22330[1]	putative cytochrome p450 (CYP79B3)	0,06	1,22	13,50	0,16	1,43	11,91
At3g08860	putative beta-alanine-pyruvate aminotransferase	153,06	0,75	0,05	36,79	0,70	0,06
At1g23730	putative carbonic anhydrase	90,51	0,89	0,13	62,44	0,87	0,10
At1g56650	PAP1 transcription factor	65,40	1,32	0,42	18,76	1,05	0,46
At1g66760	MATE efflux family protein, putative	24,06	1,22	0,13	13,18	0,86	0,16
At2g44460[2]	putative beta-glucosidase	363,51	0,75	0,03	551,89	0,77	0,01
At3g60140	beta-glucosidase-like protein	527,41	0,74	0,03	341,44	0,71	0,01
At5g48850[2]	unknown protein	818,53	0,40	0,07	161,77	0,29	0,05
At4g31330[2]	unknown protein	35,74	0,61	0,24	21,15	0,49	0,21
At5g37980[2]	quinone oxidoreductase -like protein	12,96	0,81	0,27	8,21	0,66	0,22
At1g04770[2]	unknown protein	17,55	0,18	0,08	9,53	0,19	0,11

Depending on the experiment the highest overexpression was observed for two thioglucosidase-like genes (*At2g44460* and *At3g60140*), involved in degradation of indole-glucosinolates and thus suggesting their rapid turnover under –S (Table 3-5). The expression of the genes from glucosinolates biosynthesis was also heavily affected, especially CYP79B3 (*At2g22330*), converting tryptophan to indole-3-acetaldioxime, known already to be S-responsive (Tables 3-4 and 3-5; Wittstock and Halkier, 2002). Expression levels of this gene dropped rapidly down when deprived with sulphate and began accumulate after S re-supply. Expression of CYP83B1 (*At4g31500*), acting downstream in the pathway and being responsible for oxidation of aromatic and tryptophan-derived aldoximes, was slightly (3,8-fold) affected under S-starvation. A single gene family, C–S lyase (SUR1) and thio-glucosyltransefrase UGT74B1 catalyse next two steps of indole-GLS biosynthesis and did not respond in our experimental conditions (Hansen and Halkier, 2005). Sulphotransefrases (ST) transfer the active sulphate group from 3'-phosphoadenosine 5'-phosphosulphate (PAPS) to acceptor molecules during the last step of indole-GLS biosynthesis. STs revealed slight down-regulation in –S conditions, while S re-supply caused even 7 to 12-fold up-regulation of the ST5c isoform, when compared to starved plants.

The MAM-1 gene (*At5g23010*), involved in the side-chain elongation of methionine, one of the precursors of aliphatic glucosinolates, was also down-regulated in S-deprived conditions, as well as the CYP79F1 (*At1g16410*), metabolizing chain-elongated methionine derivatives, and CYP83A1 (*At4g13770*), metabolizing in the next step the aliphatic aldoximes.

Table 3-5 Relative expression levels of selected genes involved in glucosinolates synthesis and degradation. Genes significantly (present calls) up- and downexpressed (10<R<0,1) under sulphur starvation are depicted in bold. CYP, cytochrom P450; S-GT, thio-glucosyltransferase, S-T, sulphotransferase.

Gene ID	Gene name	1st Experiment			2nd Experiment		
		-SvsFN	30'Svs-S	3hSvs-S	-SvsFN	30'Svs-S	3hSvs-S
Glucosinolates biosynthesis							
At5g23010	MAM-1	0,03	0,59	11,50	0,04	1,24	15,52
At1g16410	CYP79F1	0,06	0,39	9,57	0,05	1,41	15,36
At1g16400	CYP79F2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
At4g13770	CYP83A1	0,03	1,05	9,70	0,06	1,20	8,80
At5g05260	CYP79A2	1,00	0,80	0,60	0,67	0,75	0,75
At4g39950	CYP79B2	0,17	1,59	9,28	0,19	1,72	7,07
At2g22330	CYP79B3	0,06	1,22	13,50	0,16	1,43	11,91
At4g31500	CYP83B1	0,26	2,83	7,20	0,26	1,72	5,09
At2g20610	SUR1	0,50	1,40	3,20	0,63	1,24	2,95
At1g24100	S-GT (UGT74B1)	0,81	1,13	1,69	0,62	1,19	2,20
At1g74100	ST5a	0,52	1,57	1,58	0,65	1,23	2,06
At1g74090	ST5b	0,42	0,82	4,04	0,48	0,86	3,14
At1g18590	ST5c	0,23	1,38	7,34	0,15	1,63	12,02
Glucosinolates degradation							
At3g60140	beta-glucosidase-like protein	527,41	0,74	0,03	341,44	0,71	0,01
At2g44460	putative beta-glucosidase	363,51	0,75	0,03	551,89	0,77	0,01
At3g14210	myrosinase associated protein	0,30	1,17	1,84	0,63	1,02	1,74

When concentrating on genes involved *sensu strictu* in amino acid biosynthesis or the provision of the immediate carbon precursors (Table 3-6) we can come to following generalisations. First of all, the number of genes showing a significantly altered expression level in at least one biological replica is rather low and comprises only 9 genes (Table 3-3). Despite that, the transcriptome analysis of genes involved in amino acid biosynthesis still provides some reasonable findings when investigating the general tendencies of being induced or reduced under sulphate-limited conditions.

The putative serine hydroxymethyltransferase (SHMT; *At1g36370*) as a central gene in serine–glycine conversion is strongly induced probably in response to the block in cysteine formation which is leading to an accumulation of the cysteine precursor, serine (Table 3-6). A chloroplastic OASTL (*At3g03630*) revealed strong (10-fold) down-regulation in 1st experiment, while in the 2nd one remained rather unaffected.

Table 3-6 Relative ratios reflecting changes in expression of genes involved in amino acid biosynthesis upon sulphate starvation and readdition experiments.

		First experiment			Second experiment		
Biosynthesis and gene		**-S vs FN**	**30' S vs -S**	**3h S vs -S**	**-S vsFN**	**30' S vs -S**	**3h S vs -S**
Arginine biosynthesis:							
At2g48140	carbamoyl-phosphate synthase	6,71	1,07	0,84	4,71	1,01	0,78
Glutamine biosynthesis:							
At3g17820	glutamine synthetase (GS1)	2,05	1,04	1,00	0,95	0,98	0,93
At5g37600	glutamine synthetase, putative	2,25	1,01	0,74	1,05	1,00	0,87
Proline biosynthesis:							
At1g54100	aldehyde dehydrogenase, putative	2,23	0,93	0,79	1,29	1,01	0,90
Asparagine biosynthesis:							
At3g47340	asparagine synthetase	0,47	0,71	0,19	0,70	0,87	1,12
At4g31990	aspartate transaminase	0,75	0,84	1,20	0,89	1,03	1,21
Homoserine biosynthesis:							
At5g21060	homoserine dehydrogenase	0,57	1,56	1,29	0,78	0,98	1,00
At4g19710	homoserine dehydrogenase	1,00	1,01	1,27	0,98	1,02	1,41
At1g31230	homoserine dehydrogenase	0,39	1,05	1,64	0,58	1,10	2,40
Threonine biosynthesis:							
At2g17265	homoserine kinase	0,85	0,89	1,34	0,81	0,91	1,30
At4g11610	homoserine kinase	1,23	1,17	0,76	0,54	1,53	1,40
At1g08700	homoserine kinase	1,23	0,80	0,91	0,95	0,89	0,93
At5g28030	threonine synthase	0,66	0,93	1,08	0,69	0,95	1,28
At4g29840	threonine synthase	1,17	0,80	1,01	1,10	1,00	1,01
At3g10050	threonine synthase	1,20	0,90	1,46	0,80	1,17	1,90
At1g72810	threonine synthase	0,73	0,62	1,83	0,76	1,03	1,71
Isoleucine biosynthesis:							
At3g19710	branched-chain aa aminotransferase (BCAT4)	0,02	1,10	14,58	0,02	1,08	19,16
Lysine biosynthesis:							
At3g02020	aspartate kinase, lysine-sensitive, putative	0,43	0,66	1,96	0,44	0,85	1,99
At1g31230	bifunctional aspartate kinase=homoserine dehydrog.	0,39	1,05	1,64	0,58	1,10	2,40
At1g14810	aspartate-semialdehyde dehydrogenase	0,42	0,91	2,12	0,54	1,15	2,22
At5g52100	dihydrodipicolinate reductase family protein	0,45	0,27	1,02	0,80	0,94	1,00

At5g46180	ornithine aminotransferase, putative	1,67	0,86	0,86	1,04	1,25	1,04
Serine and glycine biosynthesis:							
At1g36370	glycine hydroxymethyltransferase, putative	228,09	0,82	0,06	19,85	0,37	0,06
Cysteine biosynthesis:							
At3g03630	cysteine synthase, chloroplast	0,10	0,19	2,54	0,65	0,72	2,07
At5g28020	cysteine synthase	1,46	0,91	0,55	2,13	0,97	0,64
At1g55920	serine acetyltransferase SAT1	2,92	1,02	0,70	2,06	0,81	0,78
Methionine and SAM biosynthesis:							
At3g01120	cystathionine gamma-synthase	0,67	0,90	1,40	0,74	0,91	1,62
At1g33320	cystathionine gamma-synthase	n.d	n.d	n.d	1,30	0,91	1,00
At4g23600	cystathionine beta-lyase	2,24	1,48	0,99	2,68	0,76	0,62
At1g64660	cystathionine beta-lyase	7,13	0,64	0,16	2,88	0,82	0,33
At3g25900	homocysteine S-methyltransferase 1 (HMT-1)	1,07	1,17	1,10	1,10	1,12	1,12
At3g63250	homocysteine S-methyltransferase 2 (HMT-2)	0,94	1,12	1,12	1,21	1,00	1,04
At3g22740	homocysteine S-methyltransferase 3 (HMT-3)	0,09	0,24	0,63	0,09	1,06	3,02
At1g02500	S-adenosylmethionine synthetase 1 (SAM1)	1,45	0,94	1,04	1,00	1,09	1,03
At4g01850	S-adenosylmethionine synthase 2 (SAM2)	0,86	0,89	1,12	0,96	1,00	1,08
At3g17390	S-adenosylmethionine synthetase, putative	1,39	0,93	0,64	1,35	0,87	0,71
At2g36880	S-adenosylmethionine synthetase, putative	1,09	1,31	1,44	0,95	1,01	1,20
Chorismate biosynthesis:							
At2g45300	3-phosphoshikimate 1-carboxyvinyltransferase	n.d	n.d	n.d	n.d	n.d	n.d
At1g48860	3-phosphoshikimate 1-carboxyvinyltransferase	1,13	0,96	1,22	1,12	1,00	1,36
Phenylalanine biosynthesis:							
At2g24850	aminotransferase, putative	1,67	1,31	0,22	0,12	0,97	0,72
At1g69370	chorismate mutase, putative	0,86	0,75	0,88	0,86	0,91	1,23
At5g22630	prephenate dehydratase family protein	2,18	1,11	0,87	1,30	1,10	0,89
At1g11790	prephenate dehydratase family protein	0,92	1,00	1,13	1,11	0,91	0,99
Tryptophan biosynthesis:							
At5g05730	anthranilate synthase, alpha subunit (ASA1)	0,96	1,00	1,60	0,63	1,08	1,80
At4g30530	GMP synthase (glutamine-hydrolyzing)	2,14	0,79	0,62	1,66	0,90	0,77
At5g17980	phosphoribosylanthranilate transferase-like protein	3,57	1,75	0,82	1,22	1,16	0,76
At5g06850	anthranilate phosphoribosyltransferase-like protein	2,31	0,94	0,98	0,72	1,03	1,60
At5g48220	indole-3-glycerol phosphate synthase	0,40	0,85	1,26	0,72	0,89	1,17
Leucine biosynthesis:							
At5g23020	2-isopropylmalate synthase 2 (IMS2)	0,25	0,88	4,92	0,31	1,02	4,08
At5g23010	2-isopropylmalate synthase-like;	0,03	0,59	11,50	0,04	1,24	15,52
At1g31180	3-isopropylmalate dehydrogenase, chloroplast	0,33	0,92	3,19	0,33	1,04	3,72
At3g58990	3-isopropylmalate dehydratase	0,11	1,35	14,23	0,13	1,01	11,23

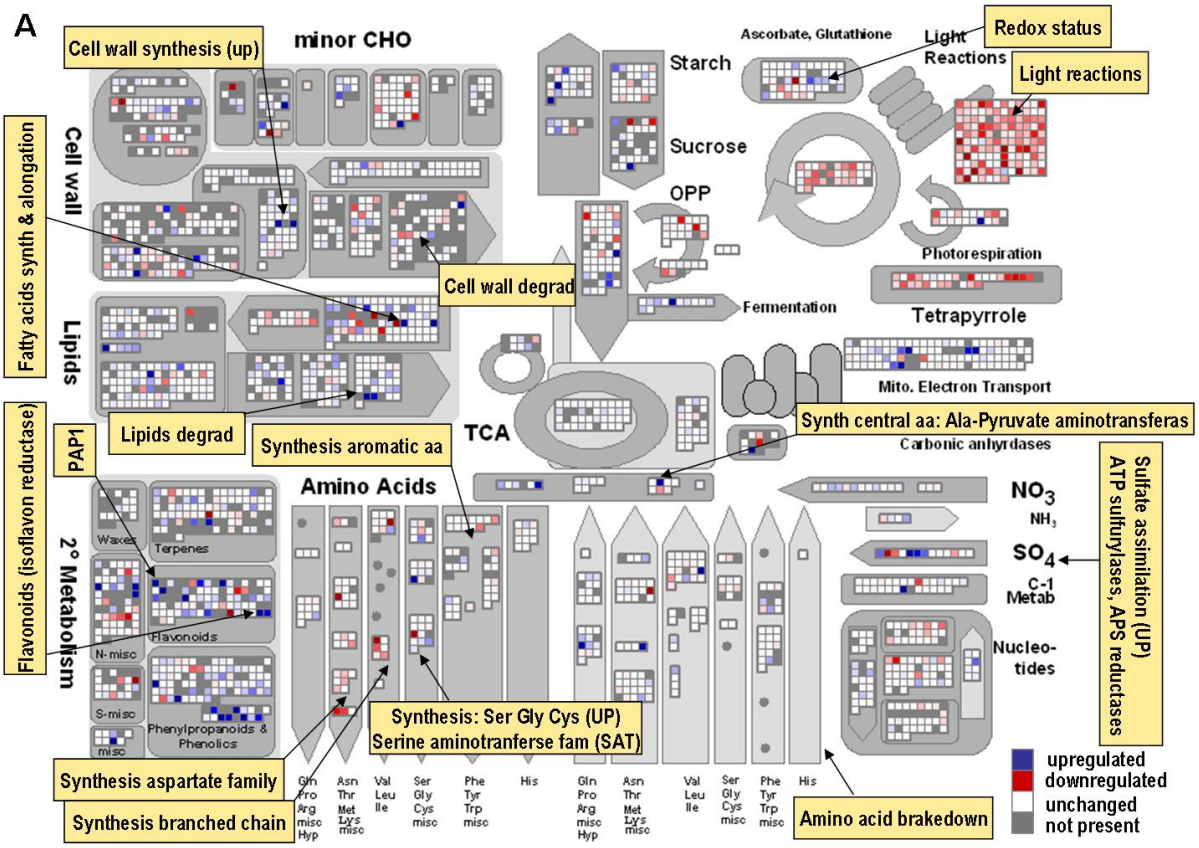
Genes involved in methionine biosynthesis are either induced, as it was observed for two isoforms of cystathionine beta-lyase (*At4g23600*, *At1g64660*) responsible for homocysteine production, or strongly down-regulated i.e. one of homocysteine S-methyltransferase 3 (HMT-3, *At3g22740*), synthesising methionine from homocysteine and S-methylmethionine (SMM). In the process of SMM-methionine interchange are involved two other isoforms of homocysteine S-methyltransferase (*At3g25900*, *AT3G63250*), these, however, remained unchanged. Also none of the genes taking part in the synthesis of S-adenosylmethionine (SAM) was changed significantly in expression in any of the conditions applied.

Other genes of the aspartate branch, to which methionine belongs, are also down-regulated such as genes for homoserine, threonine, isoleucine (BCAT4, *At3g19710*) and lysine biosynthesis.

As nitrogen assimilation is known to be impaired by S starvation (Hesse et al., 2004a; Kopriva and Rennenberg, 2004), it is of interest to detect that genes of glutamine synthesis are induced (in the 1st experiment, where the induction was stronger) to increase the sink binding capacity for N when provision of other sinks is impaired by starvation. However, asparagine biosynthesis genes remain on average unaffected. Other genes of the α-ketoglutarate branch, such as those involved in arginine biosynthesis carbamoyl-phosphate synthase (*At2g48140*), are significantly induced under sulphate limited conditions, while aldehyde dehydrogenase (*At1g54100*) from the proline biosynthesis pathway is over-expressed 2 times only in the 1st experiment.

Genes of tryptophan biosynthesis are on average induced under sulphate deprivation corresponding to tryptophan accumulation (see chapter 3.A.2.4). Transcriptional data for phenylalanine synthesis did not provide a consistent picture. All genes involved in leucine biosynthesis appeared to be down-regulated in sulphur limited conditions.

To demonstrate simultaneously the obtained results, the data visualization tool *MapMan* was used (http://gabi.rzpd.de/projects/MapMan/ Thimm et al., 2004; Usadel et al., 2005;). The whole set of data from experiment 1 was used. The changes were expressed relative to those in S-deficient seedlings. The ratios were converted to a log2 scale, and imported into *MapMan*, which converts the data values to a false colour scale. Transcripts called 'absent' are shown as grey, transcripts that change by less than a given threshold are white, transcripts that increase are blue, and transcripts that



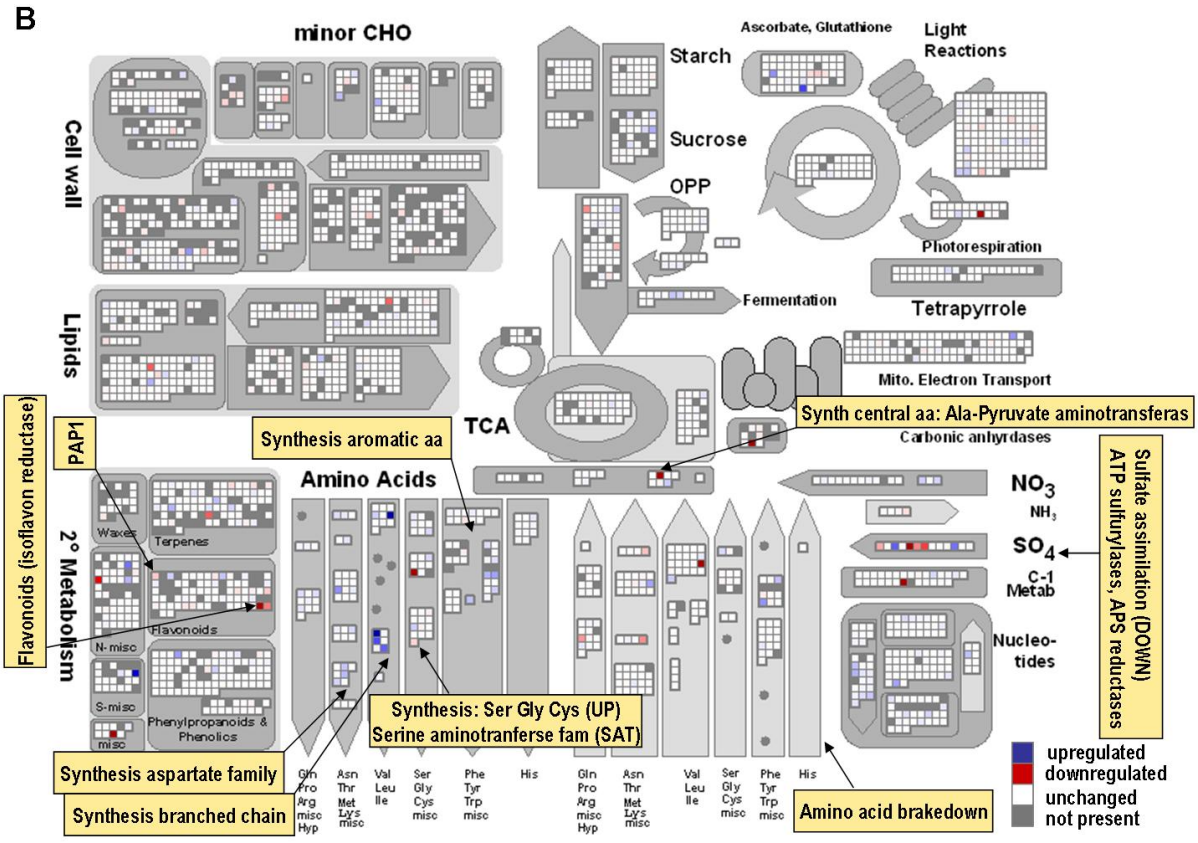


Fig 3-7 Visualisation of transcript levels in S-deficient seedlings relative to the level in S-sufficient seedlings **(A)** and in S-deficient seedlings 3h after SO_4^{2-} readdition relative to the transcript levels in S-deficient seedlings **(B)**. All results are shown on a log2 scale. The results are displayed using the *MapMan* software (Timm et al., 2004). The whole set of data from Experiment 1 was used. Genes that are called absent by Affymetrix software are shown as grey, genes that do not change by more than a threshold value as white, and genes that increase and decrease by a increasingly blue and red coloration, respectively.

decrease are red. In the scale used in figures 3-7 A and B, a 2-fold change was required to produce a visible coloration, and the scale saturated at an 8-fold (53 on a log2 scale) change.

In general, stronger changes in transcript levels were observed in plants after 48 hours of sulphur starvation, when compared to the level in S-sufficient seedlings (Fig 3-7 A), than after 3h of SO_4^{2-} re-addition in S-deficient seedlings relative to the starved plants (Fig 3-7 B). Beside the changes in transcript abundancies of genes from sulphate uptake and assimilation, amino acid metabolism or secondary metabolism, as was discussed above, plants grown in S-deficient medium exhibited a general down-regulation of genes involved in light-reactions, but also some changes in lipid metabolism and cell wall synthesis and degradation. An overview of the metabolism of re-supplied seedlings revealed strong response of most genes from sulphate uptake and assimilation pathway, already after 3 hours (Fig 3-7 B). This response manifested in coordinated changes in expression of S-responsive genes. The response of most genes strongly induced or reduced under S-limited conditions was attenuated or even reversed, which is demonstrated as opposite coloration of spots on figure 3-7 B, compared to figure 3-7 A. At the same time, many genes grouped in other metabolic bins also revealed similar reaction to increased SO_4^{2-} in medium, indicating their sulphate-specificity.

3.A.4 Expression profiling of transcription factors library

Following confirmation that the various S–treatments resulted in expected changes in expression of control marker genes and metabolite levels, quantitative RT-PCR was performed for all 1465 TF genes.

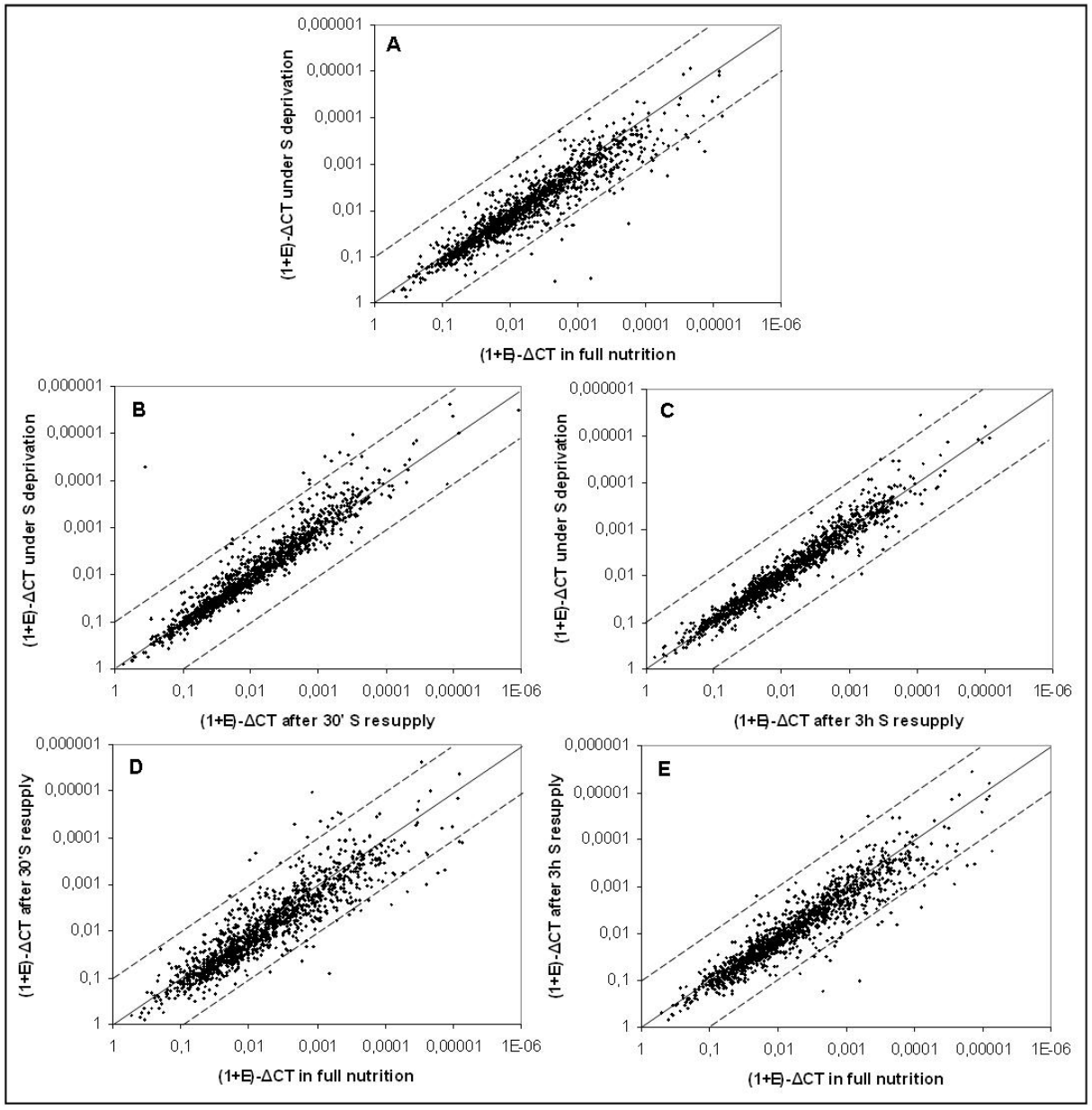


Fig 3-8 Comparison of TF transcript levels under S deprivation and 30 minutes and 3 hours after sulphate re-supply. Expression values ($(1+E)^{-\Delta CT}$) normalised to *ACT2*, from qRT-PCR amplification of cDNA from plants grown on full nutrition medium and 48 hours of sulphate deprivation (A) are depicted for 1417 TF genes resulting in specific amplicons. Expression values from plants 30 minutes and 3 hours after sulphate re-supply are plotted versus values for seedlings under 48 hours of sulphate deprivation (B, C) and versus plants grown on full nutrition medium (D, E). Dashed lines indicate 10-fold differences in the -S to FN transcript levels and in the 30 min and 3h S to -S or FN transcript levels, on pictures A-E, respectively.

Transcripts of 1417 TF genes were detectable in at least one condition analysed. Comparison of transcriptional changes for this subset of TF genes under S-deprivation and sulphate replenishment is depicted in figure 3-8. Transcript levels for *Arabidopsis* TF genes, represented by $(1+E)^{-\Delta CT}$, varied over 6 orders of magnitude. The highest TF expression level was close to that of the house keeping genes (*UBQ-10* and *ACT-2*) and the lowest just on the limit of detection of 1 transcript per 1000 cells (Czechowski et al, 2004).

To limit the number of genes for further studies, we chose a 5-fold cut off to identify S-regulated TF genes. Expression analysis for 112 TF genes selected in this way are shown in appendix C. Sixty three TF genes were found to be induced more than 5-fold after S deprivation. The most abundant group was the MYB-family with 18 TF genes up-regulated 5 times, 9 genes belonged to the MADS family, 6 to each of WRKY, AP2-EREBP and NAC families. C2H2 had 5 TF, bHLH had 4 (Fig 3-9 A). Nineteen of them exceeded a threshold level of factor 10 including TF genes from the following families: MYB (5), AP2/EREBP (3), WRKY (3), bHLH (2), C2H2 (1), MADS (3), bZIP (1) and ARR B (1). Only thirteen genes were found to be repressed more than 5-fold after 48 h of S-deprivation. The most abundant was the bHLH family, comprising 4 members (Fig 3-9 B). Of these 13 down-regulated TF genes, only one, the MYB-family TF, exceeded the threshold 10.

Fewer TF genes, from those not being previously altered significantly by –S, responded to sulphate re-addition: just one was induced more than 5-fold after 30 minutes and three after 3 hours (Appendix C). On the other hand, 32 genes were down-regulated more than 5-fold, by sulphate in those experiments, 25 after 30 minutes and 7 after 3 hours of S re-supply.

It should be noticed that some of the changes in expression are only minimal, as they result from no transcript detection in one or more experimental points (Ct value 40). When comparing the transcript abundance in one condition to 'no transcript' in another condition, the relative ratio of transcript levels presents only a theoretical value.

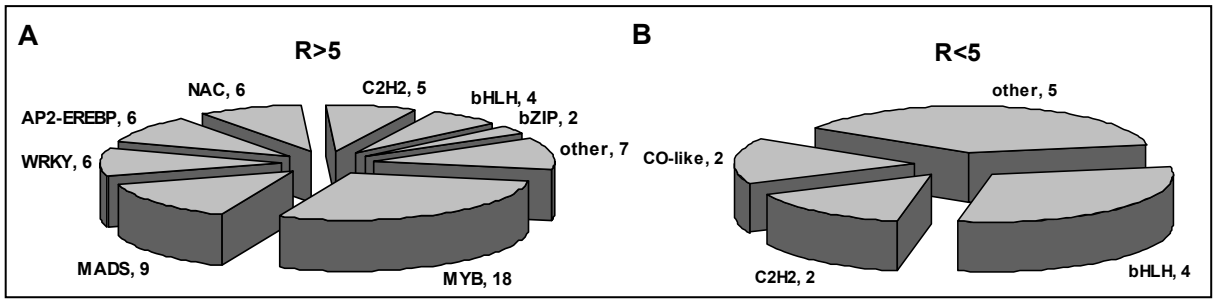


Fig 3-9 Distribution of sulphur-regulated transcription factors selected by qRT-PCR. Expression of 63 TF genes was induced more than 5-fold (**A**), while the expression of only 13 TF genes appeared to be repressed more than 5-fold under S-limited conditions (**B**). Figures represent number of members of each TF family present in the diagram.

3.A.4.1 Comparison of technologies: qRT-PCR versus Affymetrix chips

We did not necessarily expect a good correlation or similar ratios between signals obtained for the levels of the individual transcripts by qRT-PCR and Affymetrix chips. Unlike quantitative RT-PCR, hybridisation-based technologies like Affymetrix chips are qualitative and there is not a strict linear

relationship between signal strength and transcript amount for different genes (Holland, 2002). Nonetheless, genes determined to be highly expressed by qRT-PCR typically yielded also high signals on Affymetrix chips.

The basic underlying dataset for comparing both technologies compiles data from the 1st experiment obtained by qRT-PCR and Affymetrix chips.

When comparing the set of genes covered by Affymetrix chip analysis and qRT-PCR platform, 1275 TF overlap between them. However, 39 of them were not detectable in any condition analysed by qRT-PCR. Comparison of TF transcript levels for the 1236 TF genes which were detectable in seedlings grown on full nutrition medium on both, the qRT-PCR platform and on the ATH1 gene array, is depicted in figure 3-10. A large majority (94,5%) of the 456 genes that were categorised as 'absent' by Affymetrix software was detected by real-time PCR, albeit at lower levels than other TF genes, as expected. Overall, there was little quantitative agreement between the two data sets for 1236 TF genes that were analysed from seedlings grown on full nutrition medium, although slightly bigger for genes yielding high signals on Affymetrix chip (categorized as "present") (Figure 3-10). Thus, the qRT-PCR appeared to be more sensitive method than the ATH1 chip hybridisation and therefore more relevant especially for expressional analysis of transcription factor genes.

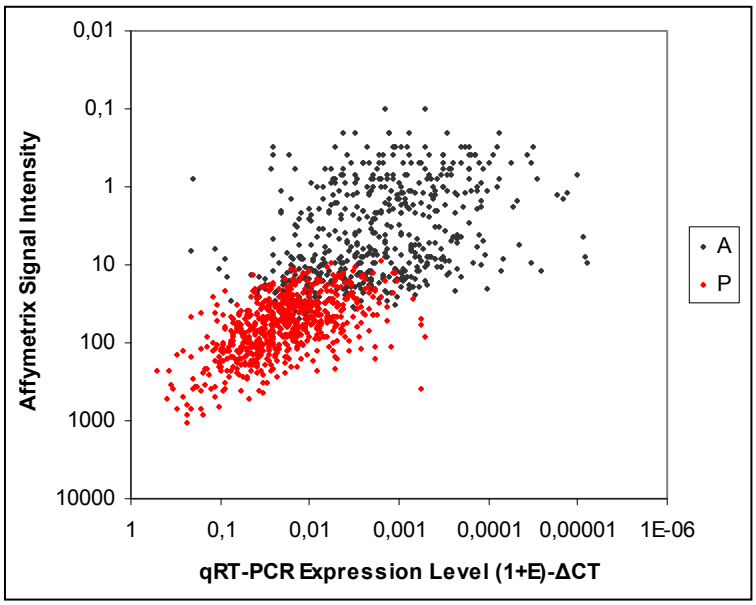


Fig 3-10 Comparison of TF transcript levels measured by qRT-PCR and Affymetrix whole genome arrays. Normalised raw data from qRT-PCR ($(1+E)^{-\Delta Ct}$) were compared to normalized raw data from Affymetrix chips (log10 of fluorescence signal) for the 1236 TF genes which were detectable in seedlings grown on full nutrition medium on both, the qRT-PCR platform and the Affymetrix ATH1 gene array. Genes categorised as 'present' or 'absent' by Affymetrix software are depicted as red or black crosshairs, respectively.

Comparison of expression ratios from ATH1 array hybridisations and quantitative RT-PCR was also used to identify S-regulated TF genes. Of the ~1800 potential TFs on the ATH1 chip, only 24 showed marked (>5-fold) changes in transcript abundance, four of them exceeded the threshold level of 10. Figure 3-11 summarizes the response (-S vs FN) for 1236 TF genes that are included in both technology platforms. Real time RT-PCR confirmed that most TF genes did not respond strongly to S

availability. Some of the genes depicted on the plots, identified as interesting only by qRT-PCR, were not identified by hybridisation Affymetrix ATH1 ('absent').

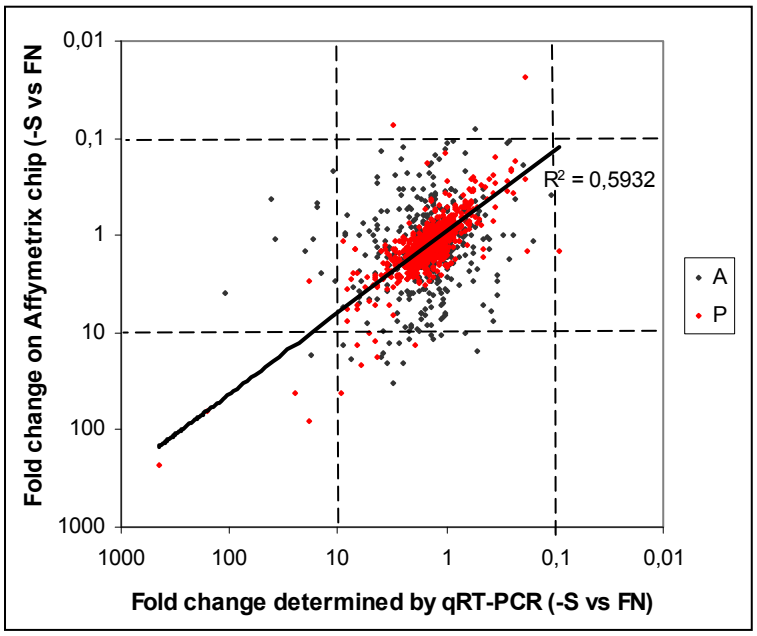


Fig 3-11 Comparison of TF gene expression ratios, as determined by qRT-PCR and Affymetrix technology. 1236 TF genes included in both platforms are shown for a comparison of S-starved versus S-sufficient *Arabidopsis* seedlings. Red and black crosshairs denote genes that were called 'present' or 'absent', respectively, on ATH1 arrays. Dashed lines indicate 5-fold changes in expression ratios. A regression line (R^2 = 0.59) is shown for the 805 'present' genes. Some genes were inconspicuous according to Affymetrix analysis, but were identified as interesting by RT-PCR.

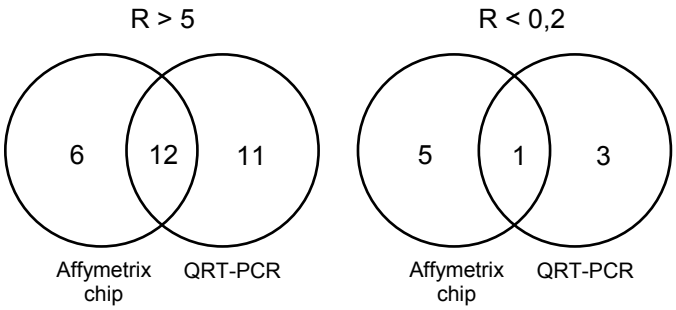


Fig 3-12 Comparative analysis of the TF gene expression patterns under sulphur deficiency stress detectable on both platforms used: qRT-PCR and Affymetrix whole genome arrays. Venn diagrams show the numbers of genes 5 times significantly up- and down-regulated in Experiment 1; R, ratio of Affymetrix chip signal intensity or normalised raw data from qRT-PCR ($(1+E)^{-\Delta Ct}$) at –S to data in full nutrition.

When considering only TF genes which were detectable on both platforms (measurable in at least one condition analysed by qRT-PCR and categorized as 'present' on Affymetrix chips), a subset of 805 TF genes was further analysed. Among these genes, eighteen were up-regulated more than 5 times on Affymetrix chips and twenty three in qRT-PCR, but only twelve of them overlapped (Figure 3-12). On the other hand, of the 23 up-regulated genes in qRT-PCR, as many as 20 exceeded the threshold level of factor 2 on Affymetrix chip. Among down-regulated TF genes, six were revealed to be sulphate-responsive on Affymetrix chips and four by qRT-PCR, but only one of them exceeded the threshold 5 on both platforms.

3.A.4.2 Reproducibility

The reproducibility of all measurements was always checked by simultaneous analysis of two experimental replicates (experiment 1 and 2). In both experiments, plants were grown in exactly the same way using the same stock of Col-O seeds.

To confirm strong S-regulation of the candidate genes mentioned above, 58 TF genes were measured with the cDNA pool coming from experiment 2 (Table 3-7). Only 14 of the 58 genes responded to S-deprivation in the same way and at similar magnitude (>5 fold change in the transcript level). 21 other genes responded in the same way but lower than the cut-off used (2-5 fold change in the expression level). Fifteen genes did not respond at the transcript level (1-2 fold change). Eight genes responded to S-starvation in the opposite way to that observed in the first experiment.

Data from ATH1 array hybridisations were also used to identify S-regulated TF genes. 20 genes, which responded similarly on both technology platforms were chosen for further comparisons (Table 3-7).

3.A.4.3 Sulphur-specificity and selection of candidate genes

A total of 20 TF genes were considered as significantly and robustly affected by sulphur deprivation. We further applied the following selection criteria to identify an S-specific subset of TF genes, which additionally would be manageable for subsequent analyses.

First, we checked whether regulation of each TF was specific to changes in sulphur nutrition, by comparing TF expression in various abiotic-stress experiments, including nitrate, phosphate, carbohydrates, osmotic and salt stress. Data were kindly provided by collaborators from the Molecular Genomic Group, MPI-MP Golm, Germany (led by Dr Wolf-Ruediger Scheible): Dr Daniel Osuna, Dr Rosa Morcuende, Rajendra Bari, and Tomasz Kobylko, and by Dr Tomasz Czechowski and Dr Wenming Zheng (from Molecular Plant Nutrition Group, MPI-MP Golm, Germany, led by Dr Michael Udvardi). All nutrient-stress experiments were done with the same axenic culture system, including the same light conditions in the same phytotron chamber, and the same basic media, except for differences in a single nutrient.

Most of the 20 S-regulated TF responded also to other stimuli, besides S-deprivation. Eleven TF responded significantly (more than 5-fold) to changes in either P and N nutrition (Table 3-8), among them *PAP1* (*At1g56650*), considered as general stress response factor as being responsible for the regulation of anthocyanin and flavonoid biosynthesis, which are activated under a variety of stresses conditions. *AtMYB107* (*At3g02940*) responded much stronger under phosphate starvation than to S-deprivation. All these genes were eliminated from further consideration with two exceptions: *At2g38340* gene from AP2/EREBP family and *AtWRKY24* (*At5g41570*). Although they responded positively to S and N deprivation, they were considered as strong candidates based on high S-induction revealed by Affymetrix hybridisation.

Table 3-7 Comparison of relative expression ratios from qRT-PCR and Affymetrix chips between both experimental replicas

AGI	Family		1st Experiment						2nd Experiment					
			qRT-PCR			Affymetrix			qRT-PCR			Affymetrix		
			-S/FN	30'/-S	3h/-S	-S/FN	30'/-S	3h/-S	-S/FN	30'/-S	3h/-S	-S/FN	30'/-S	3h/-S
At1g56650	MYB	1	164,24	0,00	0,49	65,40	1,32	0,42	19,53	0,66	0,48	18,76	1,05	0,46
At2g46130	WRKY	2	24,84	1,02	0,89	42,38	0,55	0,85	4,88	1,38	1,59	2,62	1,13	1,28
At2g38340	AP2 EREBP	3	18,41	1,01	0,48	82,67	0,43	0,03	3,63	1,13	0,58	11,75	4,49	0,53
At3g02940	MYB	4	17,97	0,27	0,57	17,00	0,48	0,11	2,78	1,18	1,31	0,69	6,09	3,18
At5g41570	WRKY	5	16,00	0,95	0,83	n.d	n.d	n.d	2,43	1,70	1,53	n.d	n.d	n.d
At1g72570	AP2 EREBP	6	15,39	0,77	1,27	0,50	2,00	3,00	3,06	0,35	unsp	2,00	0,25	0,50
At1g30670	bHLH	7	12,14	1,44	1,95	n.d	n.d	n.d	unsp	unsp	unsp	n.d	n.d	n.d
AT5g46830	bHLH	8	10,65	0,23	0,31	3,00	0,67	2,67	77,49	0,59	unsp	1,50	6,00	6,33
At1g34670	MYB	9	9,47	1,08	0,95	41,65	1,13	1,05	2,59	0,94	1,73	3,01	1,24	1,69
At1g17950	MYB	10	8,14	0,59	0,92	5,85	0,91	0,71	4,28	0,56	0,99	7,60	1,37	0,92
At1g73410	MYB	11	7,43	0,50	0,82	2,74	0,93	0,85	2,40	1,04	1,26	2,11	1,08	0,97
At1g69560	MYB	12	7,17	0,85	1,04	3,38	0,37	2,48	3,45	0,72	1,71	1,10	1,06	0,70
At1g01720	NAC	13	6,78	1,06	0,47	5,18	1,24	0,56	2,02	1,03	0,74	2,15	1,07	0,71
At5g16770	MYB-like	14	6,62	0,59	0,62	13,71	1,00	0,66	2,46	0,96	0,93	3,12	0,85	0,65
At5g65230	MYB-like	15	6,59	0,48	0,53	5,65	1,02	0,82	2,61	1,05	0,90	2,18	0,78	0,76
AT4g09820	bHLH	16	6,38	1,83	1,14	2,33	1,07	0,50	3,64	0,73	1,24	4,33	0,77	0,46
At4g33960	MADS new	17	5,15	0,48	0,18	9,40	0,48	0,20	10,36	0,38	0,21	7,77	0,48	0,25
At3g57600	AP2 EREBP	18	0,36	2,02	2,70	0,51	1,53	1,29	0,33	1,18	2,79	0,72	0,85	1,16
At1g22130	MADS redo	19	0,198	0,78	1,41	0,19	1,25	2,50	0,22	1,62	1,40	0,32	3,00	2,16
AT3g56970	bHLH	20	0,187	0,17	5,99	n.d	n.d	n.d	0,16	0,61	0,96	n.d	n.d	n.d

Table 3-8 Quantitative RT-PCR results of various abiotic stresses for sulphate regulated TF genes

AGI	Gene name	Family		Sulphate			Nitrate [1]			Phosphate [2]			Carbohydrates [3]			Mannitol [4]		NaCl [4]	
				-S/FN	30'/-S	3h/-S	-N/FN	30'/-N	3h/-N	-P/FN	30'/-P	3h/-P	-C/FN	30'/-C	3h/-C	Mann 30'/FN	Mann 3h/FN	NaCl 30'/FN	NaCl 3h/FN
At1g56650	AtMYB75	MYB	1	164,24	0,00	0,49	35,86	1,18	n.d	6,89	0,56	1,08	0,77	2,38	22,12	1,48	15,35	1,45	12,81
At2g46130	AtWRKY43	WRKY	2	24,84	1,02	0,89	8,39	2,51	n.d	1,43	1,02	1,29	0,10	1,59	5,31	0,40	4,05	1,98	2,71
At2g38340		AP2 EREBP	3	18,41	1,01	0,48	20,87	0,31	n.d	0,43	2,49	2,52	0,32	1,88	2,15	1,37	3,68	1,70	9,01
At3g02940	AtMYB107	MYB	4	17,97	0,27	0,57	0,65	1,81	n.d	0,25	1,06	1,06	47,9	1,92	6,51	0,51	4,19	1,46	3,17
At5g41570	AtWRKY24	WRKY	5	16,00	0,95	0,83	9,38	1,41	n.d	2,32	0,84	0,82	0,06	1,61	5,09	0,51	6,22	2,25	2,81
At1g72570		AP2 EREBP	6	15,39	0,77	1,27	2,77	0,79	n.d	1,23	2,52	2,65	0,07	2,05	3,54	0,47	1,11	0,96	1,18
At1g30670	AtbHLH052	bHLH	7	12,14	1,44	1,95	0,22	1,83	n.d	0,60	1,02	1,02	0,18	1,13	1,03	0,54	0,38	0,62	0,32
AT5g46830	AtbHLH028, ERF2	bHLH	8	10,65	0,23	0,31	0,75	1,49	n.d	2,05	1,21	1,08	0,24	1,17	3,74	0,65	0,81	0,62	0,33
At1g34670	AtMYB93	MYB	9	9,47	1,08	0,95	1,96	1,99	n.d	1,86	0,89	1,25	0,15	1,39	3,10	0,54	2,66	1,50	1,85
At1g17950	AtMYB52	MYB	10	8,14	0,59	0,92	3,89	1,94	n.d	3,13	0,63	1,19	0,41	1,08	2,54	0,45	4,50	1,05	2,25
At1g73410	AtMYB54	MYB	11	7,43	0,50	0,82	3,27	1,69	n.d	1,90	0,71	0,89	0,16	1,67	4,86	0,34	1,77	0,84	0,92
At1g69560	AtMYB105	MYB	12	7,17	0,85	1,04	3,71	0,75	n.d	5,11	0,86	1,07	0,31	1,15	1,88	0,57	4,56	1,04	2,15
At1g01720	ATAF1	NAC	13	6,78	1,06	0,47	3,03	2,13	n.d	0,77	0,99	1,38	1,66	1,38	0,73	2,38	2,30	3,61	3,09
At5g16770	AtMYB9	MYB-like	14	6,62	0,59	0,62	5,00	0,32	n.d	2,11	0,57	0,66	2,45	1,80	1,04	0,00	0,00	0,00	0,00
At5g65230	AtMYB53	MYB-like	15	6,59	0,48	0,53	3,68	0,48	n.d	4,37	0,90	0,82	13,2	0,97	1,25	0,00	0,00	0,00	0,00
AT4g09820	AtbHLH042, TT8	bHLH	16	6,38	1,83	1,14	5,52	1,03	n.d	2,99	0,87	1,45	0,25	1,38	3,87	0,68	2,56	0,90	1,82
At4g33960		MADS new	17	5,15	0,48	0,18	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
At3g57600		AP2 EREBP	18	0,36	2,02	2,70	0,31	1,48	n.d	1,13	2,31	1,81	0,34	1,87	1,31	0,58	0,55	1,32	0,91
At1g22130		MADS redo	19	0,198	0,78	1,41	0,09	0,76	n.d	0,12	1,22	0,95	1,03	0,54	0,05	0,64	1,43	0,93	0,28
AT3g56970	AtbHLH038	bHLH	20	0,187	0,17	5,99	0,12	3,35	n.d	0,09	0,07	0,04	2,20	4,47	9,21	1,13	1,03	0,84	0,79

Table legend:

FN – full nutrition medium

-S/FN – expression after sulphate starvation versus full nutrition ,

-N/FN – expression after nitrate starvation versus full nutrition ,

-C/FN – expression after sucrose starvation versus full nutrition ,
-P/FN – expression after phosphate starvation versus full nutrition
30'/-S – expression 30 min. after sulphate re-addition versus sulphate starvation
3h/-S – expression 180 min. after sulphate re-addition versus sulphate starvation
30'/-N – expression 30 min. after sulphate re-addition versus sulphate starvation
3h/-N – expression 180 min. after sulphate re-addition versus sulphate starvation
30'/-C – expression 30 min. after sucrose re-addition versus sucrose starvation
3h/-C – expression 180 min. after sucrose re-addition versus sucrose starvation
30'/-P – expression 30 min. after phosphate re-addition versus phosphate starvation
3h/-P – expression 180 min after phosphate re-addition versus phosphate starvation
Man 30'/FN – expression 30min after mannitol addition to FN medium,
Man 180'/FN – expression 180min after mannitol addition to FN medium,
NaCl 30'/FN – expression 30min after NaCl addition to FN medium,
NaCl 180'/FN – expression 180min after NaCl addition to FN medium,

Data kindly provided by:

[1] Tomasz Czechowski (Molecular Plant Nutrition Group, MPI-MP Golm, Germany),

[2] Dr Wenming Zheng (Molecular Plant Nutrition) and Rajendra Bari (Molecular Genomics Group, MPI-MP Golm, Germany),

[3] Dr Daniel Osuna Jimenez (Molecular Genomics Group, MPI-MP Golm, Germany),

[4] Dr Rosa Morcuende and Tomasz Kobylko (Molecular Genomics Group, MPI-MP Golm, Germany),

We selected a set of 8 genes that represents possibly specific and strong responses to sulphate deprivation: *AtWRKY43* (No 2), *At2g38340* (AP2/EREBP; No 3), *AtWRKY24* (No 5), *At1g72570* (AP2/EREBP; No 6), *AtbHLH052* (No 7), *WRKY93* (No 9), *ATAF1* (No 13) and *At4g33960* (No 17).

3.A.5 Functional characterisation of the S-regulated TF genes using knock-out mutants

For the functional characterisation of selected S-regulated TF genes a loss of function approach was taken. Database searches provided appropriate T-DNA knock-out lines, for which homozygous plants were selected and checked for a strong decrease in expression of the gene of interest.

Arabidopsis mutants impaired in selected S-regulated TF genes were obtained from SALK T-DNA insertion collection, which comprises 88 000 lines covering 21700 genes (http://signal.salk.edu/tabout.html). SALK lines were obtained as described before (Alonso et al. 2003). The database was screened via a web accessible graphical interface: T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress). Preference in choice was given to mutants with insertions in exons to maximise the chance of complete loss of the gene function.

For those genes, which were not covered by the SALK T-DNA mutant collection, a second resource, the RIKEN *Arabidopsis* transposon mutant collection was searched (http://www.brc.riken.go.jp/lab/epd/Eng/). RIKEN BioResource Center comprises 17668 single copy *Ds* transposon lines (Kuromori et al., 2004; Ito et al., 2005). The database of the insertion sites and mutated genes (http://rarge.gsc.riken.jp/) is searchable via the web accessible graphical interface: T-DNA Express (http://signal.salk.edu/cgi-bin/tdnaexpress). Unfortunately none of selected TF genes was covered by existing mutants at that time.

3.A.5.1 Selection of homozygous T-DNA KO lines

Of the 8 selected TF candidate genes, 4 were found to be covered by existing SALK T-DNA mutants with insertions in their exon regions, often more than one insertion line per gene was identified. For one TF candidate, the only existing T-DNA location was found in its 5'UTR region. Seeds for all interesting T-DNA lines were ordered from SALK collection, as mentioned above, and the screening procedure for homozygous mutant plants was performed.

Homozygous mutant plants were identified by PCR with primers that distinguished between wild type and mutant alleles (Materials and Methods chapter 2.3.7; for primer sequences see appendix E). A typical result for PCR-screening of a T-DNA mutant line is shown on figure 3-13, which clearly shows the difference between wild type (lanes "wt"), heterozygous (lanes "Ht") and homozygous (lanes "Hm") of mutant individuals. For each PCR reaction the Col-0 control was simultaneously performed. PCR reaction done on gDNA isolated from Col-0 plants resulted in a single band product, always corresponding to the wild type loci, as expected (Figure 3-13).

For some screened SALK mutant lines, only wild type-like plant individuals lacking the T-DNA insertion, were identified. Despite that, for all 5 TF genes, at least two homozygous plant individuals for one and the same SALK mutant line were found, allowing to obtain more reliable results in subsequential studies. For one gene, the WRKY24 TF, 4 homozygous lines (2 SALK lines x 2 homozygous plant each) were studied. Table 2-2 (Materials and methods, chapter 2.2.1.3) summarises all studied SALK T-DNA mutants lines.

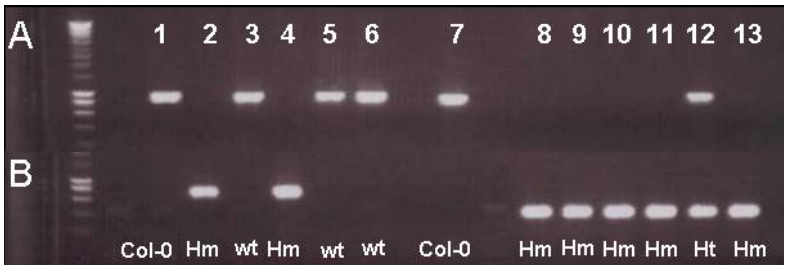


Fig 3-13 PCR screening for the homozygous T-DNA insertion line for SALK_144950 (k.o. line 3-1 for *At2g38340*) and SALK_119740 (k.o. line 5-1 for *At5g41570*). Genomic DNA was prepared from 5 individual plants from line SALK_144950 (lines 2-6), from 6 individual plants from line SALK_119740 (lines 8-13) and from Col-0 wild type (lines 1 and 7) used as a template for PCR reaction with T-DNA specific (right) or gene-specific (left) primer combinations as described in Materials and Methods. wt – wild type loci, Ht – loci heterozygous for T-DNA insertion, Hm – loci homozygous for T-DNA insertion.

3.A.5.2 Loss-of-function test of target genes

The T-DNA mutant SALK lines (T4 generation) were cultivated using the same liquid culture system as it has been used for the initial experiment (chapter 3.A.1). RNA from all selected homozygous SALK mutant lines (see above) was isolated and cDNA synthesis was performed.

Suitable primer pairs for qRT-PCR specific to the given target gene were designed. Special effort was taken to design these primers on the sequence located downstream to the T-DNA insertion point, otherwise an aborted PCR product could have been detected, giving artificial, misleading results (for primer sequences see appendix E).

No transcript or at least strong repression of transcription was detected for following plant lines: 5-1-2, 5-1-7, 5-4-18, 5-4-2, 9-1-1, 9-1-9, 17-1-8, 17-1-3 (Table 3-9). Although the process of morphological characterisation and the metabolite screening will be demonstrated for the wider set of SALK T-DNA mutant lines than those, for which the loss of gene function was proved, all further conclusions about the function of each TF gene studied in this approach should be driven form the results achieved for lines: 5-1-2, 5-1-7, 5-4-18, 5-4-2, 9-1-1, 9-1-9, 17-1-8 and 17-1-3, exclusively.

Table 3-9 Relative expression levels between T-DNA insertion SALK mutants and wild type plant calculated for the T-DNA insertion target genes. RNA was isolated from axenicly grown T4 generation SALK mutant lines. The given primer pairs were designed on following gene sequences: *At2g38340* (3), *At5g41570* (5), *At5g46830* (8), *At1g34670* (9), *At1g01720* (13), *At4g33960* (17). Values depicted in bold indicates significant differences between mutant and Col-0 lines.

cDNA from plant line	Primer pair ID	Ct	ΔCt	ΔΔCt	SALK line vs Col-0
3-1-2	3	29,22	11,05	-0,16	1,12
	3	29,38	11,21	-0,44	1,36
3-1-4	3	28,87	11,55	0,34	0,79
	3	29,25	11,93	0,28	0,83
5-1-2	5	35,37	17,70	10,28	**0,001**
	5	35,98	18,31	10,11	**0,001**
5-1-7	5	33,46	15,65	8,23	**0,003**
	5	34,62	16,81	8,61	**0,003**
5-4-18	5	34,70	16,09	8,66	**0,002**
	5	34,96	16,35	8,15	**0,004**
5-4-2	5	28,82	10,12	2,70	**0,15**
	5	28,80	10,10	1,90	**0,27**
8-3-17	8	33,86	15,83	0,06	0,96
	8	33,61	15,58	0,15	0,90
8-3-18	8	n.d	n.d	n.d	n.d
	8	34,62	16,64	1,21	0,43
9-1-1	9	38,51	20,31	10,67	**0,001**
	9	36,87	18,67	9,03	**0,002**
9-1-9	9	34,46	16,57	6,92	**0,01**
	9	n.d	n.d	n.d	**n.d**
13-2-1	13	24,05	6,03	0,72	0,61
	13	24,00	5,98	0,70	0,62
13-2-2	13	24,05	5,18	-0,13	1,09
	13	24,32	5,45	0,17	0,89
17-1-3	17	30,09	12,34	2,39	**0,19**
	17	30,10	12,35	2,40	**0,19**
17-1-8	17	32,20	14,38	4,43	**0,05**
	17	32,16	14,34	4,39	**0,05**
Col-0	3	29,00	11,21		
	3	29,44	11,65		
	5	25,21	7,42		
	5	25,99	8,20		
	8	33,56	15,77		
	8	33,22	15,43		
	9	27,43	9,64		
	9	27,43	9,64		
	13	23,10	5,31		
	13	23,07	5,28		
	17	27,74	9,95		
	17	27,74	9,95		

3.A.5.3 Growth phenotypes and root architecture studies

None of the selected homozygous KO lines from table 2-2 gave an aberrant phenotype when grown under standard conditions in the greenhouse. Neither germination nor flowering time of any mutant line differed from those observed for Col-0 plants (data not shown).

Mutants were also screened for changes of root architecture as described in Materials and methods (chapter 2.2.2.3). None of the KO mutant lines showed changes in root architecture compared to WT controls (data not shown).

3.A.5.4 Content of elemental sulphur and other elements in seeds

A preliminary study of the content of elemental sulphur stored in SALK mutant seeds was performed. The T5 generation of seeds from several plant individuals, being offsprings of one and the same homozygous T4 parent plant, was pooled. Elemental sulphur content for each SALK line and Col-0 line was measured using ICP-AES method (IACR-Rothamsted, Harpenden, UK). Thereby, the seed storage capacity of selected T4 generation homozygous lines was investigated. Due to plant material limitation, only one measurement of T5 generation seeds and none of T4 generation seeds, was performed.

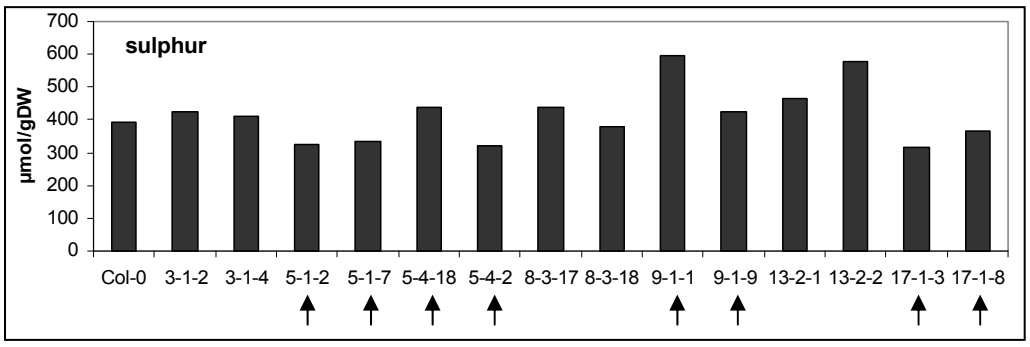


Fig 3-14 Content of elemental sulphur in T5 generation of SALK knock out lines. Arrows indicate lines, for which the loss of gene function was proven.

Slight fluctuations in sulphur content for the T5 generation of SALK knock out lines were observed (Fig 3-14). The highest difference to the Col-0 line revealed line 9-1-1, which contained 150% of wild type level of sulphur. Unfortunately, the second homozygous SALK line did not confirm this result, containing a sulphur amount comparable to Col-0. The lowest sulphur levels were obtained for the pair of homozygous lines, 5-1-2 and 5-1-7 (83 and 84% of wild type sulphur level, respectively), selected for SALK_119740 (5-1). However, another SALK line with a T-DNA insertion in the same gene (*WRKY24*), did not give confirming results. Only one homozygous line, 5-4-2, revealed lowered sulphur level (82% of wild type sulphur level), the other, 5-4-18, contained a sulphur amount comparable to Col-0. Other lines, for which the loss of gene function was confirmed, 17-1-3 and 17-1-8, also showed lower sulphur levels, compared to wild type, but differed between each other (81 and 92%, respectively). For all the lines tested, the general conclusion can be driven, that the T-DNA insertion in the target gene does not correspond to the changes of sulphur level.

Among other elements, calcium and natrium exhibited some changes, but these changes did not correspond to the knock out effect of the target genes, either (data not shown).

3.A.5.5 Measurement of thiols and sulphate content in SALK-lines – investigating the physiological phenotype

Due to an absence of visible phenotypes in all of the selected homozygous SALK knock out lines, it was of special interest to investigate the contents of those metabolites, which could give some evidence for a physiological phenotype. It was expected, that our candidate TF genes, selected as sulphur-reacting, will affect the sulphur metabolism, when impaired. As thiols represent the major sulphur components and immediate products of sulphate assimilation, determination of thiol levels was assumed to reveal the metabolic responses of plants to the changes caused internally by plant's transcriptome.

Analysis of the amounts of thiols in SALK mutant seedlings grown in full nutrition medium did not provide any evidence for phenotype changes in most of those lines, when compared to wild type plants. In most cases, high variability in the thiol levels for each two homozygous T4 lines derived from one and the same T3 SALK line did not allow to drive any meaningful conclusion (Fig 3-15).

A consistent result was obtained for the lines 5-1-2, 5-1-7 and 5-4-18 (all containing T-DNA insertion in the *WRKY24* gene), in which the level of cysteine was decreased to 62, 71 and 78% of wild type plants, respectively. However, the homozygous line 5-4-2 did not reveal the same tendency, rather showing a significantly higher cysteine level (159% of Col-0 level). This misleading result could be, however, correlated with an incomplete loss of function of the target gene (see table 3-9). Both lines 5-1-2 and 5-1-7 revealed decreased levels of cysteine also in S-deficient conditions, 73 and 60% of that of Col-0, respectively (Fig. 3-15). Homocysteine levels in the lines 5-1-2 and 5-1-7 were slightly lowered under control conditions (81 and 84% of the Col-0 content, respectively), while under sulphur starvation, homocysteine content was on average 2-fold lower than in wild type plants (59 and 44% of wt levels, respectively). Similar results were obtained when analysed γ-Glu-Cys and gluthathione content in the lines 5-1-2 and 5-1-7. γ-Glu-Cys decreased significantly (66 and 57% of wt levels) under sulphur limited conditions, while a significant decrease of gluthatione was observed in both conditions. 82 and 83% of the Col-0 level of glutathione was detected in control conditions and 67 and 51% of the wild type level of glutathione was detected under S-limited conditions, in the lines 5-1-2 and 5-1-7, respectively.

In contrast to that, lines 9-1-1 and 9-1-9 presented elevated amounts of thiols, when compared to wild type (Fig. 3-15). The cysteine level was significantly higher under S-limited conditions, determined as 156 and 135% of the Col-0 content. Also γ-Glu-Cys increased significantly in S-starved seedlings of the lines 9-1-1 and 9-1-9, being respectively 2 and 1,5 times higher than in wild type. The highest increase was detected for homocysteine, which was 1,7 times higher in both lines grown on full nutrition medium, compared to Col-0 and, 3,8 and 2,9-fold higher than in wild type under –S in the lines 9-1-1 and 9-1-9, respectively.

In all of the lines, transgenic and wild type, the level of all four thiol-compounds decreased significantly after 2 days of sulphur starvation, when comparing to the corresponding plants grown on sulphur-sufficient medium (Fig 3-15). However, for many lines, in which the complete loss of gene function was not confirmed, the levels of thiols were obtained significantly different from those of Col-0, which complicates correlation between loss-of-function of TF gene and the thiol levels.

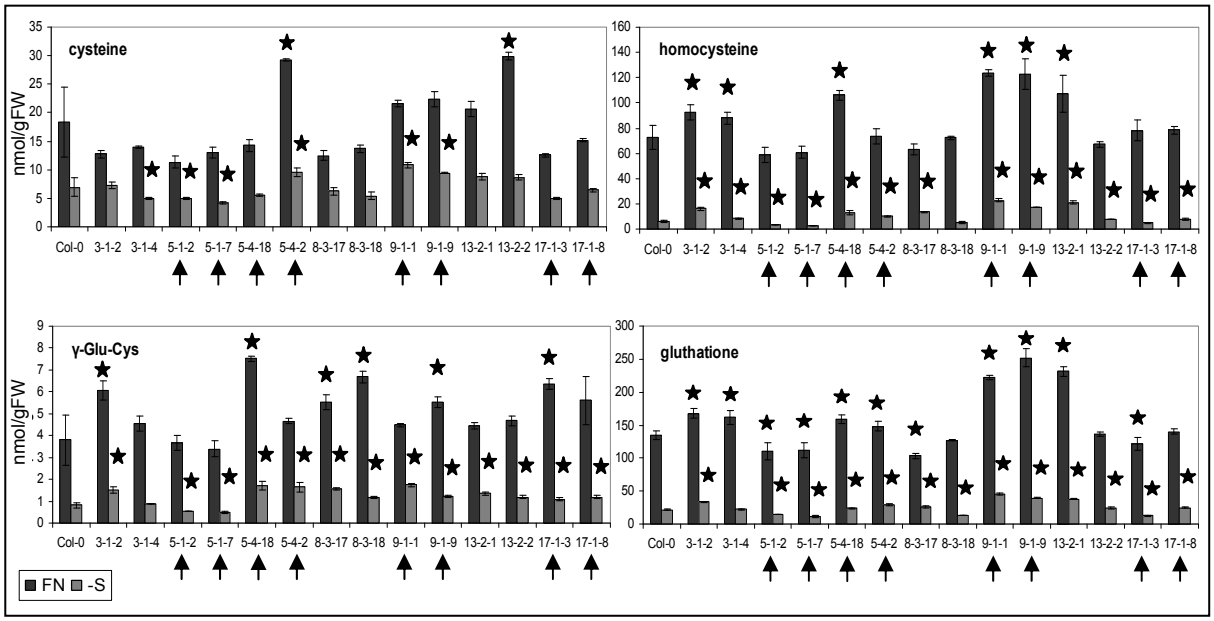


Fig 3-15 Levels of thiol-compounds in seedling of T4 generation SALK mutant lines grown in liquid cultures under different sulphur regimes. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different (P < 0,05) in comparison to the respective FN controls. Arrows indicate lines, for which the loss of gene function was proved. (FN, full nutrition; -S, sulphate deficient medium).

The content of internal anions, such as sulphate, nitrate and phosphate was determined to see, whether the impaired genes influenced the nutrient status of SALK mutant plants. As it is depicted in figure 3-16, the sulphate level was rather constant within lines and varied from 86 to 116% of that of wild type level, when considering plants grown in full nutrition medium. Among lines, for which the complete loss of the gene function was confirmed, the significant decrease (86% of sulphate content in wild type) was determined in lines 9-1-1 and 17-1-3, for plants grown in control conditions.

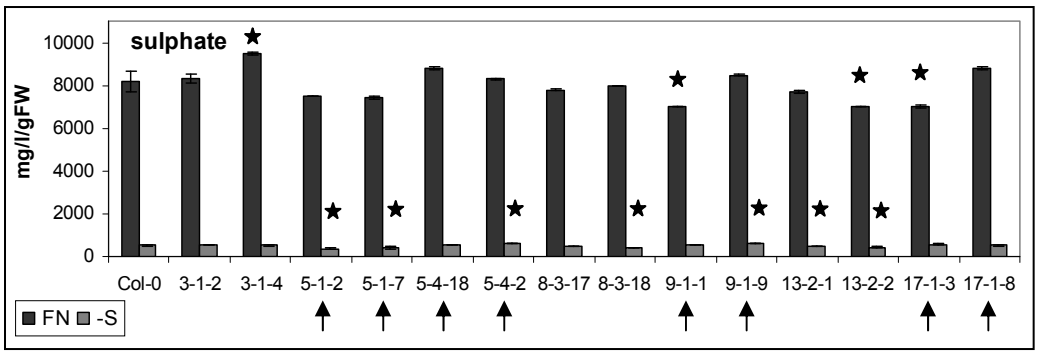


Fig 3-16 Sulphate content in seedling of T4 generation SALK mutant lines grown in liquid cultures under different sulphur regimes. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different (P < 0,05) in comparison to the respective FN controls. Arrows indicate lines, for which the loss of gene function was proved. (FN, full nutrition; -S, sulphate deficient medium).

After 48h in zero-sulphate medium, the internal sulphate level dropped significantly down in all lines tested (Fig. 3-16). In Col-0 lines sulphate decreased to 6,6% of those of full nutrition plants (15-fold). Stronger decreases in internal SO_4^{2-} content revealed lines 5-1-2 and 5-1-7 (21 and 17-fold), which resulted in SO_4^{2-} amounts determined as 66 and 79% of that of starved Col-0 plants. Sulphate amounts slightly higher than in starved wild type plants were detected in lines 5-4-2 and 9-1-9, which under –S accumulated 113% of wild type SO_4^{2-} level.

Phosphate and nitrate levels remained rather constant despite the S-starvation and did not differ considerably between transgenic and wild type lines (data not shown).

3.A.5.6 Searching for regulated elements – expression patterns of S-regulated genes in SALK-lines

Genes involved in sulphate metabolism and others known to be S-reactive were considered as the most likely biological target of our TF candidate genes. Target expression analysis on SALK T-DNA insertion lines using quantitative RT-PCR revealed increased transcripts levels of some genes from sulphate uptake and assimilation, especially (Table 3-10; for primer sequences see appendix E). Among sulphate transporters, highest transcript abundancies were found, surprisingly, for the SULTR 3;5 (*At5g19600*) and SULTR 3;2 (*At4g02700*), which are not known to be induced by sulphur starvation. Both low affinity sulphate transporters, SULTR 2;2 (*At1g77990*) and SULTR 2;1 (*At5g10180*) showed increased expression levels in knock out lines. Interestingly, AtSULTR 3;5 is suggested to function as a heterodimer with AtSULTR 2;1 (Kataoka et al., 2004a). Among the high affinity sulphate transporters, only the SULTR1;2 (*At1g78000*) was up-regulated in 5-4-2, 5-4-18, 9-1-1 and 9-1-9 lines, while *SULTR 1;1* expression remained unaffected in all of the SALK mutants lines. Only one gene, the *SULTR 1;3*, was found to be down-regulated in all transgenic lines tested. Among the genes acting downstream in the pathway, the groups of ATP sulphurylases and OAS (thiol)lyases were revealed to be over-expressed in most of the tested lines. Interestingly, genes belonging to the APS reductase family were down-regulated exclusively in lines 17-1-3 and 17-1-8, while in others were mainly unchanged. Genes which are known to be strongly reacting upon S-deprivation (Hirai and Saito, 2004), did not reveal stronger than 3-fold induction in any of the SALK mutant lines, except for *CYP79B3* gene, which increased 3,18 times n the line 5-4-18.

When comparing the changes in expression levels between all SALK knock out lines tested, both homozygous lines of SALK_008183 (5-4-2 and 5-4-18; insertion in *WRKY24*, *At5g41570*) and both of SALK_131752 (9-1-1 and 9-1-9; insertion in *MYB93*, *At1g34670*) were indicated as the most affected. However, other lines with T-DNA insertion in the *WRKY24* gene, 5-1-2 and 5-1-7, revealed induction in almost only OASTL gene family. It is worth mentioning, that two different TF genes, WRKY24 and MYB93, occurred to act similarly, at least at the transcriptional level of the set of S-reactive genes investigated.

Table 3-10 Relative expression level of genes involved in primary sulphate assimilation pathway measured for axenicly grown T4 generation SALK mutant lines, for which the loss of gene function was proved. Ratios (SALK k.o. vs Col-0) higher than 2.0 are depicted in bold, red – up-regulated, black – down-regulated.

AGI code	Gene name	5-1-2	5-1-7	5-4-18	5-4-2	9-1-1	9-1-9	17-1-3	17-1-8
At4g08620	Sultr1;1	1,28	1,47	0,94	0,83	1,34	0,66	1,29	1,13
At1g78000	Sultr1;2	1,61	1,87	**3,96**	**2,03**	**3,49**	**2,85**	0,98	1,51
At1g22150	Sultr1;3	**0,39**	**0,35**	**0,18**	**0,39**	**0,29**	0,61	**0,36**	**0,19**
At5g10180	Sultr2;1	1,08	0,89	**3,49**	1,50	**2,35**	**2,06**	1,04	1,23
At1g77990	Sultr2;2	1,74	1,82	**5,37**	**3,95**	**4,47**	**2,99**	1,83	1,46
At3g51895	Sultr3;1	1,07	1,27	**2,01**	1,44	1,18	1,28	0,98	0,93
At4g02700	Sultr3;2	1,36	1,14	**3,45**	**2,52**	**2,79**	**2,79**	1,24	1,31
At3g15990	Sultr3;4	0,92	0,86	1,42	1,17	1,10	1,60	0,55	0,95
At5g19600	Sultr3;5	1,29	1,23	**7,85**	**3,63**	**4,32**	**3,92**	1,27	1,67
At5g13550	Sultr4;1	1,90	1,58	1,07	1,26	2,01	1,63	1,10	1,29
At3g12520	Sultr4;2	0,83	1,00	**2,64**	1,92	**2,28**	1,79	0,77	1,33
At1g80310	Sultr5;1	1,44	1,29	1,60	1,73	1,72	1,25	1,01	1,24
At2g25680	Sultr5;2	0,88	0,84	**3,76**	**2,29**	**3,30**	**2,61**	1,95	1,22
At3g22890	ATPS1	**2,00**	**2,38**	**4,67**	**2,69**	**3,70**	1,75	1,52	1,46
At1g19920	ATPS2	1,55	1,88	1,84	**2,06**	**2,96**	**2,42**	1,60	1,98
At4g14680	ATPS3	1,71	1,38	**4,63**	**2,85**	**3,13**	1,56	1,44	1,37
At5g43780	ATPS4	1,20	1,14	1,04	1,33	1,14	0,95	0,90	1,00
At2g14750	APSK1	1,35	1,44	**2,68**	1,93	**2,30**	1,25	1,38	1,06
At4g39940	APSK2	1,89	1,90	**2,93**	**2,34**	**3,26**	**2,02**	**0,22**	1,38
At4g04610	APR1	1,19	1,06	1,83	0,63	1,66	1,28	0,50	**0,18**
At1g62180	APR2	1,47	1,63	**2,52**	1,24	**2,81**	1,16	1,24	**0,20**
At4g21990	APR3	1,10	0,91	1,41	0,69	1,30	1,04	**0,34**	NM
At1g55920	SAT 1	1,68	1,18	1,95	1,49	1,87	**2,68**	1,26	1,16
At3g13110	SAT A	0,51	0,59	1,28	0,70	1,24	0,79	0,61	0,74
At5g56760	SAT 52	1,28	1,07	1,85	1,72	1,58	1,05	1,22	0,94
At2g17640	SAT2 106	**2,81**	1,90	**5,21**	**5,41**	**5,92**	**2,69**	1,97	1,56
At4g14880	1OASTLA1	**2,75**	**2,44**	**11,74**	**4,51**	**10,11**	0,88	1,62	NM
At2g43750	2OASTLB	1,09	1,10	**4,07**	**2,00**	**2,40**	1,66	1,13	0,54
At3g04940	5OASTLD1	**7,06**	**5,35**	**22,79**	**17,86**	**21,33**	**10,57**	**3,52**	**0,31**
At3g22460	6OASTL	**3,24**	**3,31**	**4,78**	**3,58**	**4,66**	**4,80**	**2,23**	0,55
At3g61440	7OASTLC1	1,52	1,35	**3,15**	**2,12**	**2,54**	1,93	1,08	0,62
At5g24660	putative protein (vikin-like1)	1,23	0,89	1,09	0,54	0,99	0,92	0,83	**0,42**
At1g75280	IFR	1,25	1,19	1,53	**2,03**	1,42	1,26	1,17	0,99
At3g05400	sugar transporter, putative	NM	0,95	1,34	1,27	**2,19**	1,62	1,11	1,23
At5g26220	ChaC-like protein fam. of cation transp.	1,67	1,18	1,79	1,37	1,54	1,37	1,15	1,90
At1g36370	Ser hydroxymethyltransferase (SHMT)	1,41	1,25	1,68	1,66	1,67	1,20	0,95	1,85
At3g44320	NIT3	1,45	1,54	**2,69**	**2,19**	**2,20**	0,88	1,35	1,30
AT1G18870	isochorismate synthase, putative	1,83	1,30	0,67	0,49	**2,56**	0,95	0,90	1,19
At1g08830	superoxide dysmutase (CSD1)	1,65	1,52	**2,05**	1,83	1,99	1,87	1,35	1,38
At1g76680	OPR1	**2,39**	1,95	**2,01**	**2,62**	**2,02**	1,34	0,88	1,07
At1g76690	OPR2	NM	**2,26**	1,96	**2,17**	**2,35**	0,83	1,13	1,14
At2g06050	OPR3	1,03	1,12	**2,20**	1,15	1,49	1,02	0,74	1,03
At1g75270	DHAR	1,23	1,56	1,91	1,33	**2,05**	1,63	0,85	1,08
At2g22330	putative cytochrome p450 (CYP79B3)	1,07	1,27	**3,18**	1,86	1,95	1,82	1,19	1,10

Different patterns of changes in expression levels were found in *At4g33960*. Both homozygous lines of SALK_149207 revealed main changes in expression of genes from APS reductase family, which were down-regulated, when compared to wild type. Except for two OASTL genes, which were slightly over-expressed in the line 9-1-1, none of the tested genes revealed induction in any of SALK_149207 line.

3.A.5.7 The *At4g33960* gene

The resulting set of putative TFs present on the qRT-PCR platform was selected based on their sequence homology to known TF domains (see chapter 2.3.3.1). However, some of these proteins may bind non-specifically to DNA. Not all of genes that were targeted are necessarily TF genes. These genes were selected because they encode DNA-binding and other domains that are shared by TF proteins, which does not necessarily mean that they are transcription factor genes.

According to BLASTP (http://www.arabidopsis.org/cgi-bin/wublast/wublast), an expressed protein encoded by the *At4g33960* gene possesses a weak homology to a floral homeotic agamous-like MADS-box protein APETALA1, encoded by *At1g69120*. The homology between both proteins was found in the TF domain region. Thus, initially, the *At4g33960* gene was considered to be a transcription factor. However, according to a recent knowledge, the molecular function of the *At4g33960* gene remains unknown, giving no evidence for its involvement in the regulation of transcriptional processes. To date, none of the *Arabidopsis* transcription factor databases includes the *At4g33960* gene.

3.B Functional characterization of two APETALA 2 TF genes selected in the late response to the sulphate starvation approach

There has been substantial progress on the elucidation of the pathways involved in sulphur uptake and assimilation in recent years. Approaches, such as transcriptomics and metabolomics aimed to reveal the signaling components of plant response to sulphur starvation. However, the central, still open question is the transduction of an information flux to provide the most effective reaction. Given the apparent importance of transcriptional regulation, components involved in transcriptional control have been described, such as potential sulphur-responsive elements in promoter regions of S-responsive genes. The next major step would be identification of *trans*-acting elements. Here we aim to link the function of two AP2 transcription factor genes with methionine metabolism and provide evidence that they may work as part of a regulatory network that regulates sulphate metabolism in *Arabidopsis*.

3.B.1 Experimental background

Both AP2 candidate genes were selected in a long term sulphur-depletion experiment in which *Arabidopsis* plants were subjected to 10 and 13 days of constitutive and to 6 and 10 days of induced sulphur-starvation. To examine the temporal expression behaviour of approximately 7200 non-redundant genes, corresponding to about 30% of the total *Arabidopsis* genome, an EST macroarray hybridization was applied (Nikiforova et al., 2003).

The experiment was designed in a way to identify statistically significant changes of gene expression based on sufficient numbers of repeated hybridizations performed with five uniform pools of plant material. Changes between transcript levels of plants grown on normal medium as a control and on sulphur-deficient medium were analysed by calculating the ratio (R) of the average transcript level from five repetitions on sulphur-deficient medium to the average transcript level from five repetitions on control sulphur-sufficient medium for each EST clone. Statistical significance of differences in expression levels was analysed with t-test. In all analyses the difference was considered significant with a probability of $P < 0.05$. For all EST clones, R more than 2.5 or less than 0.4 with $P < 0.05$ at least in one of the four experimental points were the criteria used to assign statistically significant differential expression. However, the expression levels and ratios for genes encoding transcriptional factors were generally lower than those of other functional groups. Therefore, slightly less strict parameters were applied to this group of genes. Thus, these genes were considered to be sulphur responsive, if R was more than 2 or less than 0.5, with $P < 0.05$ at least in one of the four experimental points.

When applying the described selection criteria to the whole dataset at all four experimental points, 1507 sulphur-responsive clones were revealed. Among them 826 EST clones totally and 80 clones referring to transcriptional factors exhibited over-expression in sulphur-deficient conditions. Respectively, 681 clones and four transcriptional factors were down-regulated during sulphur starvation. However, among the 1507 sulphur-responsive clones implicated in this way, only 632

genes responded specifically to sulphur deficiency by significant over-expression. In order to distinguish between general stress response and the specific response to sulphur deficiency, the data were compared to data obtained under other stress conditions, like: iron deficiency (Thimm et al., 2001) and herbicide treatment (unpublished data). After applying these filters, 67 transcription factor genes were identified, which assumingly responded specifically to sulphur deficiency by significant over-expression, among them two APETALA2 genes: *At2g28550* (*TOE1*) and *At5g60120* (*TOE2*). As it is shown in table 3-11, the expression of the *At2g28550* TF gene was 2,86 times elevated after 6 days of induced sulphur starvation in all five replicas, when compared to control, while the *At5g60120* TF gene responded significantly after 10 days of constant sulphur deficiency being 2,31 times higher expressed than in normal conditions.

Table 3-11 Changes in expression of two APETALA 2 TF genes in long-time starvation experiment in four experimental points (Nikiforova et al. 2003). In the experiment 1 seeds were sown directly on sulphur deficient medium and seedlings were starved for 10 (Exp 1.1) and 13 days (Exp 1.2). In the experiment 2 seeds were first pre-grown on normal medium (8 days), then seedlings were transferred to –S medium for 6 (Exp 2.1) and 10 days (Exp 2.2). Expression changes are shown in ratios of average intensity at –S to average intensity at normal S. Significant changes are depicted in bold.

AGI code (gene name)	Construct number	Exp 1.1	Exp 1.2	Exp 2.1	Exp 2.2
At2g28550 (*TOE1*)	# 63	0,60	0,53	**2,86**	1,31
At5g60120 (*TOE2*)	# 70	**2,31**	1,81	0,69	1,11

To further investigate the function of selected AP2 TF candidate genes, they were tested under long term sulphur-depletion in an reverse genetic approach, using *Arabidopsis* over-expressing lines. By comparing metabolites and transcript profiles from transgenic and wild type plants it was expected to confirm the role of these TF genes in regulation of sulphate metabolism.

The gain of function approach involved cloning of selected TF genes for constitutive over-expression driven by 35S promoter, plant transformation and selection of the transgenic lines showing increased expression of the gene of interest. The selection of T1 plants containing constitutive TF constructs: *35S-At2g28550* and *35S-At5g60120* was based on Northern blot analysis (see chapters: 2.3.7, 2.3.8, 2.3.10). T2 generation seeds were harvested from all selected T1 over-expressing *Arabidopsis thaliana* plants and were kindly provided by co-workers (see chapter 2.2.1.2).

3.B.2 Level of overexpression in T2 generation plants confirmed by quantitative RT-PCR

cDNA from T2 lines containing the *35S-At2g28550* construct (further termed # 63) and lines containing the *35S-At5g60120* construct (further termed # 70) was used as a template for qRT-PCR reactions. Two sets of reactions were done: with primer pairs designed for *At2g28550* gene and for *At5g60120* gene (for primer sequences see appendix E). For plants grown in normal S conditions, *At2g28550* gene expression level was on average 3 times higher in leaves and on average 6 times higher in roots in T2 lines containing *35S-At2g28550* construct, when compared to wild type, thus confirming overexpression of the *At2g28550* gene in respective transgenic plants (Fig 3-17, panel A).

Expression of this gene in line 70-30 containing *35S-At5g60120* construct was on the wild type level, as expected (panel A). An opposite result was obtained, when using a primer pair designed for the gene *At5g60120*. For plants grown in +S medium, 13,5 times higher expression level was detected for the line 70-30 in leaves and 7,6 times higher in roots, while the lines # 63 show the *At5g60120* gene expression level in both tissues comparable to wild type (Fig. 3-17, panel B). Sulphur depletion did not change significantly the elevated expression levels of the studied genes. In leaves, sulphur limitation caused a slight increase of the *At2g28550* expression level in the line 63-10 (from 2,3 up to 3,2-fold), the *At5g60120* level in the line 70-30 was increased as well (up to 15-fold). In roots, under –S conditions the *At2g28550* and *At5g60120* transcript abundances reached over 13-fold in the lines 63-5 and 70-30, respectively, in comparison to Col-0 level. In the lines 63-10 and 63-12 expression level of the *At2g28550* gene slightly decreased. In general, the *At2g28550* over-expression was higher in roots than in leaves, while the *At5g60120* ratio of expression exhibited opposite tendency for tissue specificity.

The selection of four APETALA2 T2 lines used for the qRT-PCR assay was based on several metabolite screenings performed prior to the qRT-PCR measurements (see chapters below). The process of morphological characterisation and metabolite screening, which resulted in a narrowed-down list of lines giving repeatable, meaningful results, will be demonstrated in following chapters for the whole set of T2 lines (initially 11 lines). Nevertheless, all further conclusions about the function of AP2 genes of interest are based and discussed on results achieved for lines: 63-5, 63-10, 63-12 and 70-30, in which the activity of exogenous AP2 TF genes has been proven by qRT-PCR.

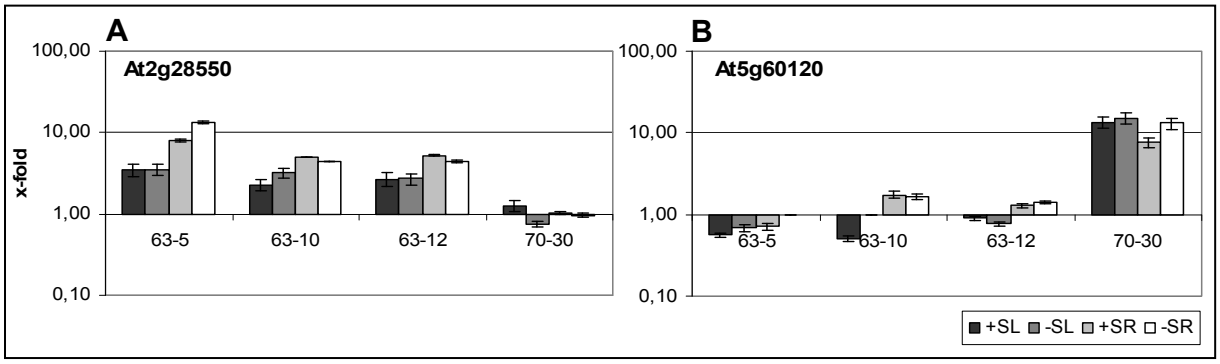


Fig 3-17 Changes in expression level of the transcription factor AP2 genes: *At2g28550* (panel **A**) and *At5g60120* (panel **B**) in T2 lines harbouring constructs: *35S-At2g28550* (line 63) and *35S-At5g60120* (line 70-30). Data are means ±SD for n = 3. Transcript abundances of these genes in transgenic lines are compared to abundances in Col-0 line grown in S-sufficient conditions. Ratio of expression levels is shown in logarithmic scale (log10). +/-SL, leaf plant material cultivated in control or S-deprived conditions; +/-SR root plant material cultivated in control or S-deprived conditions.

3.B.3 Morphological characterisation of selected transgenic AP2 lines

Transgenic 35S:AP2 plants displayed common features in their phenotype, significantly different to Col-0 plants, when grown on soil in greenhouse conditions. Phenotypes of both generations, T1 and T2, were studied and documented. However, the root system of soil-grown plants

is inaccessible for photo-documentation, thus to investigate the root system architecture, a set of vertical plate experiments was performed.

3.B.3.1 Soil-growth phenotype

Visible phenotypes were recorded while T1 plants were grown in the greenhouse. Figure 3-18 shows the phenotypic variation on 4 exemplary T1 plants of each line.

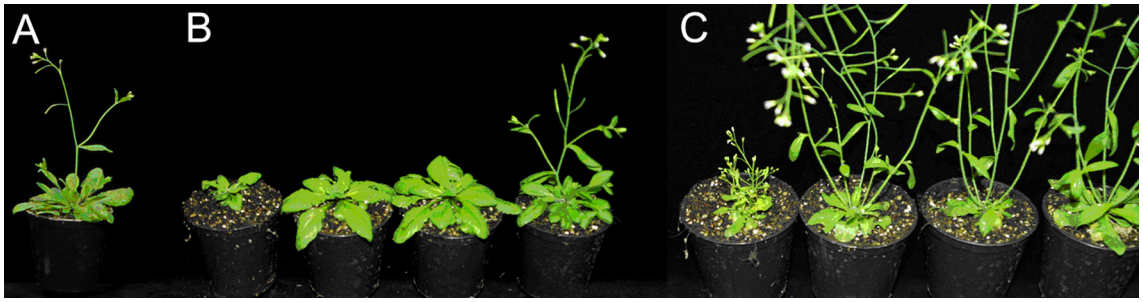


Fig 3-18 Phenotypic variation of 4 exemplary T1 plants of line 63 (containing *35S-At2g28550* construct) (**B**) and line 70 (containing *35S-At5g60120* construct) (**C**), compared to the wild type plant (**A**).

The majority of T1 plants containing *35S-At2g28550* construct (# 63) were strongly delayed in flowering and showed large rosette leaves. This phenotype was also stable in the T2 generation (selected late-flowering plants from T1 produced also late-flowering T2 progeny). The late-flowering phenotype segregated also in the T2 generation, where about 20% of the plants looked like wild type, some had never formed an inflorescence.

T1 plants containing *35S-At5g60120* construct (# 70) also yielded significantly altered phenotypes. Most of the plants were flowering earlier than wild type and had smaller rosette leaves. About 10% of T1 plants showed a strongly stunted growth. Those dwarfed plants produced small amounts of seeds when grown under standard greenhouse conditions. This phenotype variation was also stable in the T2 generation.

Figure 3-19 presents the phenotype variation within the T2 generation lines, compared to wild type Col-0 plants (panel A) and Col-0 transformed with the empty vector (panel B). As for the parental line, mature *35S-At2g28550* T2 plants (# 63) exhibited strong delay in flowering, especially the lines 63-10 (panel E) and 63-12 (panel G). For delayed or non-flowering plant individuals, an increased number of curly rosette leaves was observed, all leaves were significantly bigger than those of Col-0, however, the total leaf number remained unchanged. The inflorescence of late-flowering T2 plants displayed floral defects (see also figure 3-20). Many of the late-flowering *35S-At2g28550* T2 transformants often displayed inflorescence alterations, such as strongly reduced overall stalk height and small number of secondary inflorescence initiated, resulting in decreased total number of flowers per plant individual (Fig. 3-20).

Mature *35S-At5g60120* transformants surprisingly exhibited opposite alterations, having smaller rosette leaves and slightly decreased total leaf number, compared to wild type plants (Fig. 3-19, panel D, F and H). Flower morphology was not affected but flower number was increased, due to the higher number of secondary inflorescences, when compared to Col-0 plants of the same age.

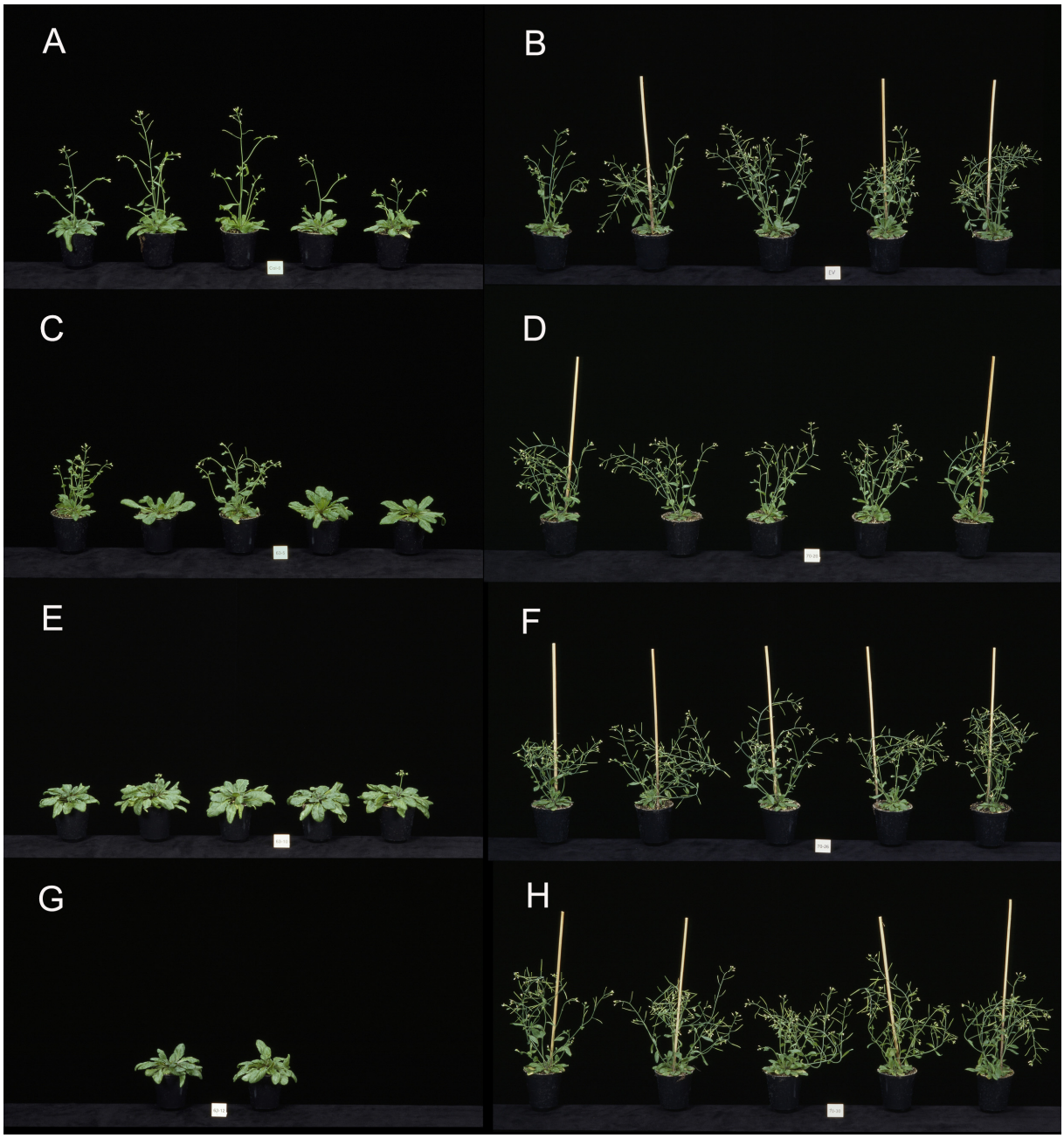


Fig 3-19 Phenotypic variation in the T2 generation of the *35S-At2g28550* harboring lines: 63-5 (**C**), 63-10 (**E**), 63-12 (**G**) and of lines possessing construct *35S-At5g60120*: 70-20 (**D**), 70-26 (**F**) and 70-30 (**H**) compared to Col-0 line (**A**) and lines transformed with an empty vector (**B**). Five plants per line were grown for a month in standard greenhouse conditions and photographed.

However, upon longer cultivation the line 70 revealed rapid development and, thus, shorter life time. After 6 weeks of cultivation, Col-0 plants were close to the end of their life time (siliques still closed) (Fig 3-20A), while *35S-At5g60120* transformants were already fully ripe and senesced (data not shown). At the same time the line 63 was still viable, however the yellowish old rosette leaves and visible antocyanin accumulation in younger leaves suggested, that the senescence process had already started in these plants. Some *35S-At2g28550* transformants were poorly developed and showed abnormal inflorescences (Fig 3-20, panel B, C and D).

Fig 3-20 Phenotypic variation in the T2 generation of lines: 63-12 (**B**), 63-5 (**C**) and 63-10 (**D**) compared to the wild type Col-0 (**A**) after long cultivation period (6 weeks).

3.B.3.2 Vertical plates

To check for additional visible phenotypes, 35S:AP2 plants were screened for changes of root architecture on vertical plates, as described in chapters 2.2.2.1 and 2.2.2.3. 7 days old seedlings were transferred from BASTA-plates (for selection of transformants) to final square plates and grown under different sulphate regimes. 5 days after transfer, first visible starvation symptoms were recorded for plants grown on zero-sulphate plates.

Up to the 8th day after transfer to the normal S condition plates, all three *35S-At5g60120* lines (# 70) exhibited accelerated growth rate, in comparison to Col-0, which resulted in increased overall size of these plants (Fig 3-21b). *35S-At2g28550* transformants started to display the growth retardation effect, when grown on +S plates; most affected was the 63-5 line (Fig 3-5a). 8 days after transfer to S-deficient conditions, starvation symptoms developed strongly for all plants grown on –S plates.

Prolonged S-starvation of up to 14 days resulted in death of all plants growing on –S plates, however the +S plates revealed interesting phenotypes of *35S-At2g28550* plants (line 63). All three lines were retarded in growth and accumulated antocyanins in leaves (Fig 3-22, panel A). Most affected was the line 63-5, which positively correlated with the level of over-expression, compared to wild type. *35S-At5g60120* plants were again still bigger in overall size than Col-0, having bigger rosette leaves and a more developed root system (Fig 3-22, panel B).

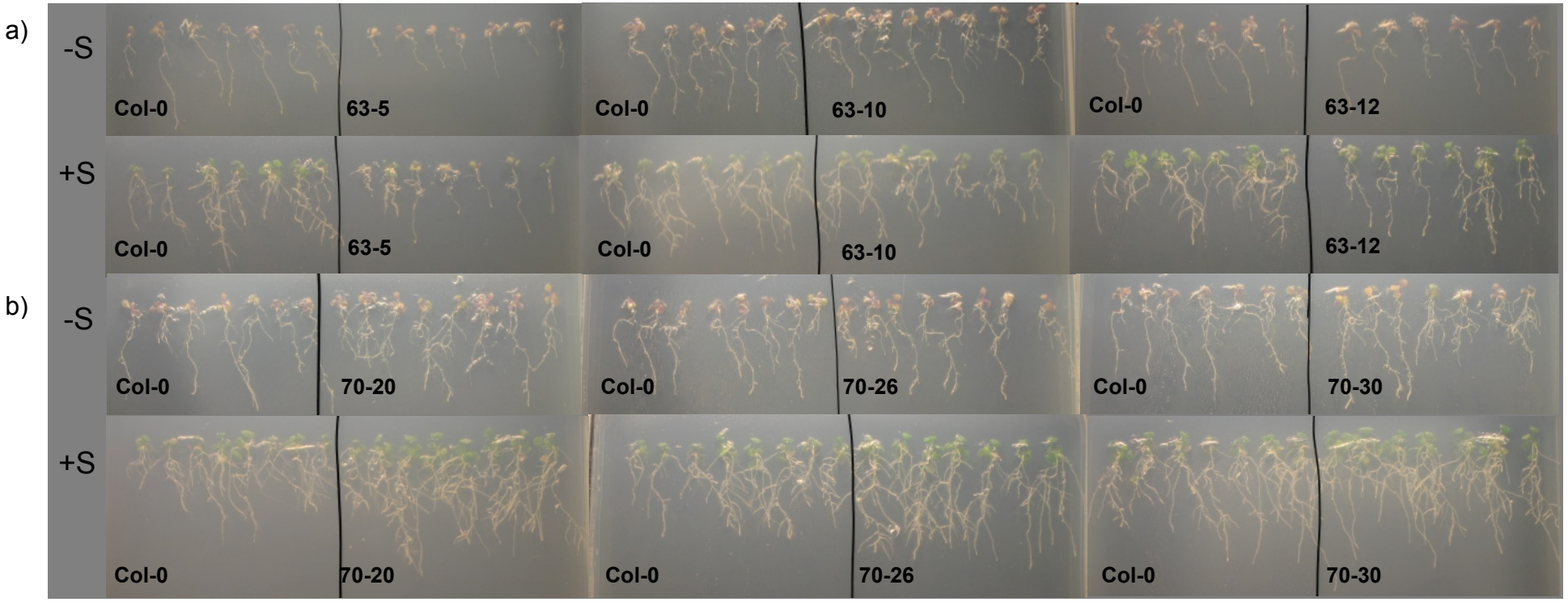


Fig 3-21 Root architecture study for *Arabidopsis* plants transformed with *35S-At2g28550* **(a)** and with *35S-At5g60120* construct **(b)** grown under different sulphure regimes: 750µM SO_4^{2-} (+S) and zero sulphate level (-S). Transgenic and wild type seedlings were grown vertically on square agar plates and photographed on the 8th day after transfer (=15 days after sowing).

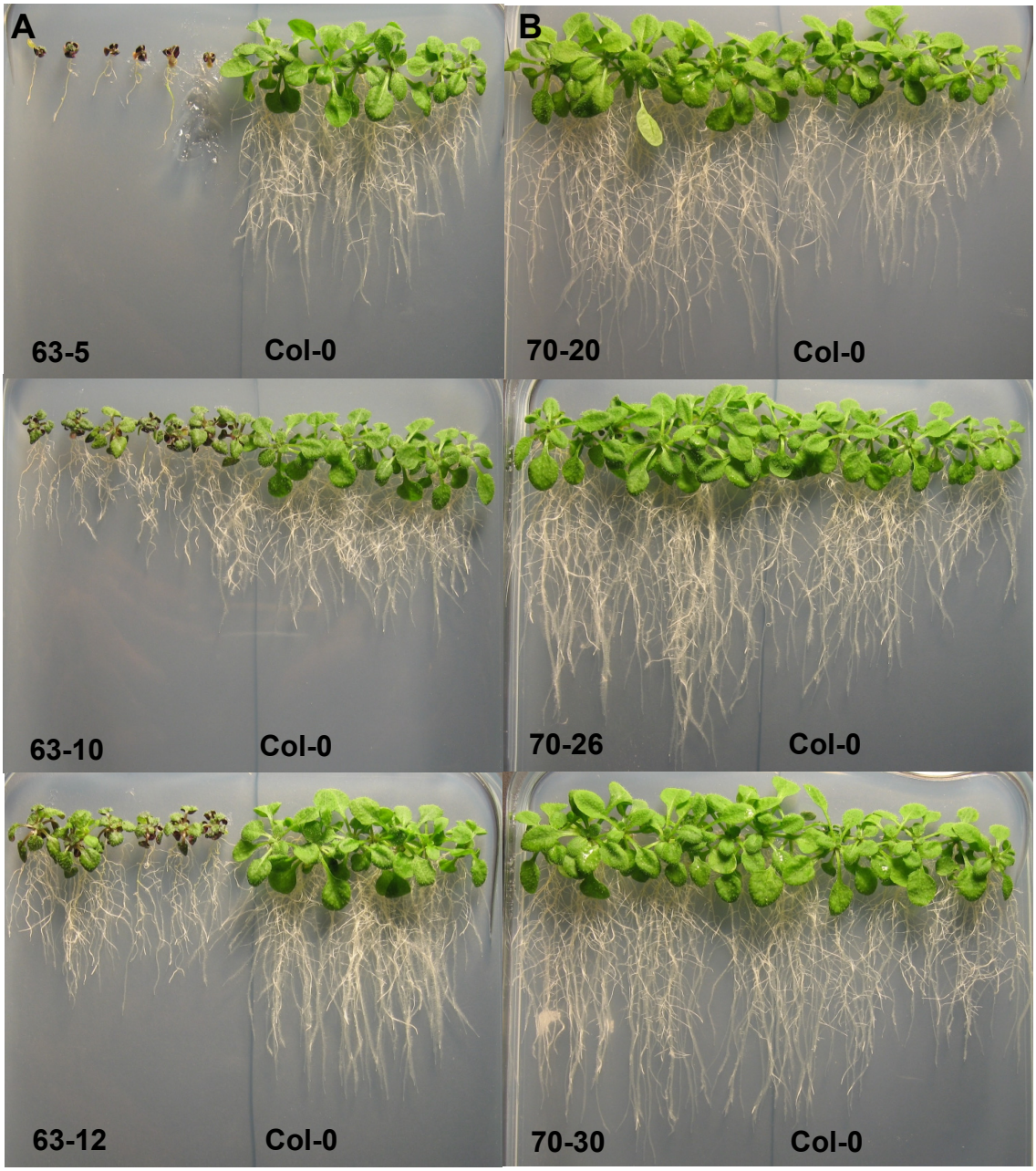


Fig 3-22 Root architecture study for *Arabidopsis* plants transformed with *35S-At2g28550* **(A)** and with *35S-At5g60120* construct **(B)** grown vertically on square agar plates for 14 days after transfer (=21 days after sowing). Photographed were plants grown on normal S medium (750µM SO_4^{2-}), since prolonged sulphur starvation resulted in plant death.

3.B.4 Metabolites

Altered gene expression of the transcription factors of interest could disturb biosynthetic pathways and, thus, manifest in changes in metabolic phenotype. To investigate a response of the entire plant system, changes in metabolites connected to sulphur metabolism were checked. It was assumed, that over-expression of both AP2 genes, selected as sulphur-reacting, will affect the sulphur metabolism.

3.B.4.1 Ions

To check, whether the nutrient status of 35S:AP2 plants was influenced by respective AP2 gene over-expression, the content of internal anions, such as sulphate, nitrate and phosphate was determined.

In plants grown in normal S conditions, the sulphate level in leaves was rather constant within lines and varied from 61 (significant decrease in the line 63-5) to 108% (line 70-20) of that of wild type level (Fig. 3-23). Changes in lines 63-12, 63-30 and 70-18, which contained 89, 84 and 88% of wild type sulphate content, respectively, were also indicated as significant. After 10 days in sulphate limited conditions, the internal sulphate level of Col-0 leaves decreased to 34% of that of normal S conditions (3-fold). Interestingly, leaves of 35S:AP2 plants revealed even stronger decreases of internal sulphate levels, after 10 days in –S medium. The lowest amount of SO_4^{2-} was found in the line 63-10, where it dropped to 13% of the SO_4^{2-} level in control S conditions (7,5-fold).

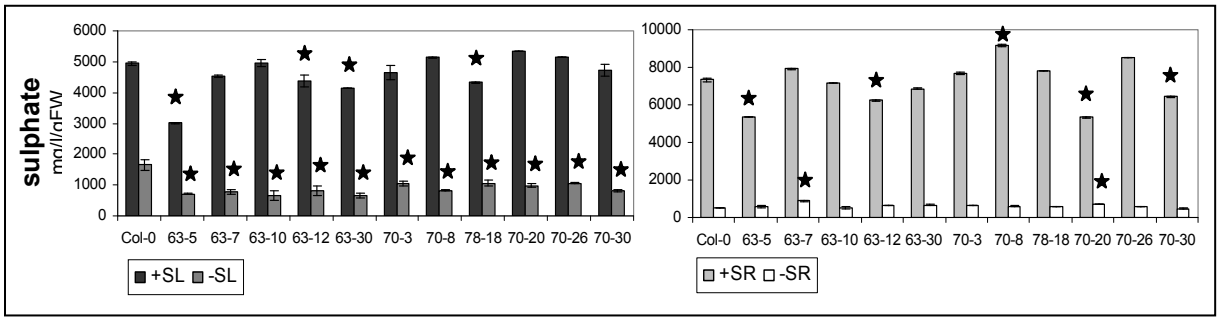


Fig 3-23 Sulphate content in 35S:AP2 plants grown in hydroponics under different sulphur regimes. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different ($P < 0,05$) in comparison to the respective Col-0 controls. +/-SL, leaf plant material cultivated in control or S-deprived conditions; +/-SR root plant material cultivated in control or S-deprived conditions.

In roots, measurements of the internal sulphate level gave similar results. In plants grown in normal S conditions, the sulphate level in roots varied from 73 (lines 63-5 and 70-20) to 125% (line 70-8) of that of wild type level, which was indicated by t-test as significantly different from Col-0 (Fig. 3-23). Significant changes were found also in the line 63-12 (85% of Col-0 SO_4^{2-} level) and 70-30 (88% of Col-0 SO_4^{2-} level). After 10 days of sulphur deprivation, Col-0 roots contained only 7% of initial sulphate amount. Differently as in leaves, after 10 days in –S, roots of most of 35S:AP2 plants contained comparable or higher amount of internal SO_4^{2-}. In two lines, 63-7 (172% of Col-0 SO_4^{2-} level) and 70-20 (139% of Col-0 SO_4^{2-} level), this difference was indicated by t-test as significant.

Nitrate and phosphate levels have been kept constant in both tissues despite the S-starvation and did not differ considerably between transgenic and wild type lines (data not shown).

3.B.4.2 Thiol determination

To investigate, whether over-expression of investigated AP2 transcription factors, induced any responses in sulphate metabolism, the level of thiols, as major sulphur components and immediate products of sulphate assimilation, was determined. It was expected to select lines, which would

considerably differ in content of thiols from wild type plants and, thus, narrow down the set of lines subjected for further studies.

Analysis of thiol amounts in 35S:AP2 plants grown in standard S conditions revealed some interesting changes in thiol levels, especially for the *35S-At2g28550* line (# 63), when compared to wild type plants. In leaves, the cysteine level was in general lower for all transformants than for control plants (Fig 3-24). The lowest level was obtained for the line 63-12, which contained only 48% of the wild type cysteine amount. Remarkably low was also the Cys level in lines 63-5, 63-10 and 70-18, having 55, 59 and 56% of Col-0 cysteine amount, respectively. The largest difference between control and transgenic plants revealed homocysteine measurements, for which the lines 63-7, 63-10, 63-12 and 63-30 displayed an over 3-fold increase, when grown in standard S conditions. On the other hand, levels of γ-Glu-Cys (GEC) and glutathione (GSH) in leaves were constant within lines and comparable to respective wild type amounts.

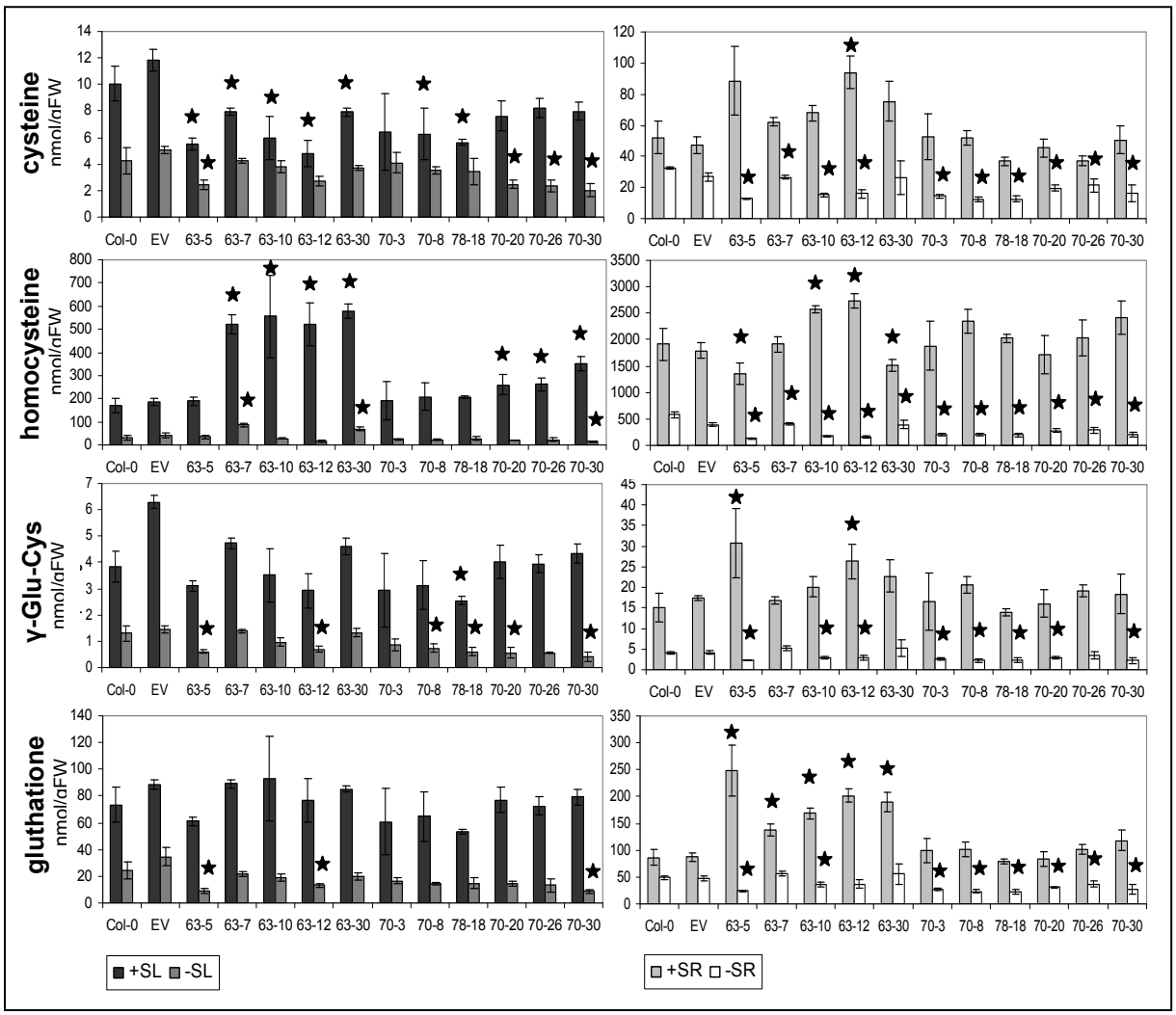


Fig 3-24 Levels of thiol-compounds in 35S:AP2 plants grown in hydroponics under different sulphur regimes. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different (P < 0,05) in comparison to the respective Col-0 controls. +/-SL, leaf plant material cultivated in control or S-deprived conditions; +/-SR root plant material cultivated in control or S-deprived conditions.

Analysis of thiol amounts in roots revealed elevated amounts of glutathione for all plants possessing the *35S-At2g28550* construct (Fig 3-24). The highest level of glutathione displayed the line 63-5, which had 287% of that of Col-0 level, line 63-12 – 232%, 63-30 – 219%, 63-10 – 194% and line 63-7 had 160% of Col-0 glutathione amount. However, the γ-Glu-Cys amounts were indicated by t-test as significantly higher only for lines 63-5 and 63-12, as having 203 and 174% of Col-0 γ-Glu-Cys level, respectively. Significantly higher amounts of homocysteine were found in leaves in the lines 63-10 (135% of Col-0 level) and 63-12 (143% of Col-0 level), while the cysteine level was elevated only in the 63-12 line (181% of Col-0 level). Leaves of other lines kept the thiol amounts on wild type level, only two lines revealed lower than Col-0 amounts of homocysteine: 63-5 (71%) and 63-30 (79%).

Following the results obtained by ion measurements, which revealed strong sulphate deprivation under applied experimental conditions (see chapter above), the thiol analysis showed decreases of all four thiol-compounds after 10 days of S starvation. In leaves, Col-0 and empty vector (EV) control plants displayed respectively a 2,4 and 2,3-fold decrease of cysteine, 5,3 and 4,6-fold decrease of homocysteine, 3 and 4,3-fold decrease of γ-Glu-Cys and 3 and 2,6-fold decrease of gluthatione (Fig. 3-24). Roots of control plants displayed slightly milder S starvation symptoms, when considering the thiol levels. After 10 days in –S, cysteine in roots dropped 1,6 and 1,8 times in Col-0 and EV, respectively, homocysteine 3,3 and 4,6 times, γ-Glu-Cys 3,6 and 4,2 times and glutathione 1,8 times in both control plants. In leaves of 35S:AP2 plants, the decrease was in general comparable with that of control plants, however, with some remarkable exceptions. The cysteine level in –S conditions was slightly lower in leaves of transformants than in control plant leaves. This resulted in lower +S/-S cysteine ratios, which might be interpreted as the tendency to keep the cysteine level in leaves as high as possible, despite the S starvation. The lowest ratio between starved and non-starved plants was obtained for the line 63-10, where the cysteine dropped only 1,5 times. An interesting situation was observed for homocysteine in the starved leaves of lines 63-10 and 63-12, where the decrease of homocysteine was stronger than in Col-0 leaves (87 and 53% of the initial amount), resulting in large +S/-S homocysteine ratios: 20 and 30-fold for 63-10 and 63-12, respectively. The similar tendency revealed line 70-30, where the homocysteine decreased in leaves 24 times after 10 days in limited sulphur conditions, γ-Glu-Cys – 10 times and glutathione decreased 9 times. When analysing the thiol amounts in roots of the starved plants, a common tendency was observed for all *35S-At5g60120* lines, that the decrease of all four thiol-compounds is significantly stronger, when compared to thiol decrease in corresponding wild type plants.

Based on data obtained for thiols, it was decided to select the following lines for further detailed studies: 63-10 and 63-12 as displaying lower cysteine amounts in leaves and accumulating higher homocysteine levels in leaves and roots and 63-5 accumulating γ-Glu-Cys and glutathione in roots. Among *35S-At5g60120* lines it was decided to concentrate on line 70-30 as displaying the strong decrease of all four thiol-compounds in both tissues under sulphur limited conditions and elevated levels of homocysteine in leaves in control conditions, when compared to changes presented by wild type plants.

3.B.4.3 Amino acids

Amino acids were the next group of metabolites, where changes induced by over-expression of selected AP2 transcription factor genes, were investigated.

As some alterations in cysteine levels were found by thiol measurements (see chapter above) in 35S:AP2 plants, other 3-phosphoglycerate derived amino acid levels were expected to be affected, as well. Indeed, serine levels were higher than in Col-0, in leaves and roots of tested 35S:AP2 plants (Fig. 3-25). For instance, line 63-10 displayed an over 3 times higher serine amount in leaves than Col-0 plants, for line 63-12 this ratio was 2,7. Serine, the precursor of cysteine, known to accumulate upon a limited input of sulphur, increased slightly in both leaves (1,24-fold) and roots (2-fold) of starved Col-0 plants, while in transformants this increase was significantly larger (Fig. 3-25). The highest serine increase was observed in leaves for the lines 63-10 (2,3-fold) and 63-12 (3-fold), in roots – 2,4 and 3,6, respectively.

In standard sulphur conditions, the level of glycine in Col-0 leaves was remarkably lower than in transgenic lines, where Gly increased 14 (63-5), 136 (63-10), 132 (63-12) and 59 times in the line 70-30 (Fig. 3-25). Under S-depletion, a concurrent accumulation of glycine occured, as closely linked to serine formation (Li et al., 2003; Bauwe and Kolukisaoglu, 2003, Nikiforova et al., 2005). In –S, it increased 12,4 times in Col-0 leaves, while for 35S:AP2 plants this increase was lower (e.g. 3-fold for line 63-10, 9-fold for 63-12), nevertheless resulted in accumulation of up to 100 times higher glycine amount than in starved Col-0 leaves. Similar tendency was observed for roots, in which the strongest glycine accumulation was detected for lines 63-12 (4,3-fold) and 63-10 (3,7-fold) in standard S conditions and the highest increase of glycine under –S (6,5 times) was detected for the starved 63-12 line.

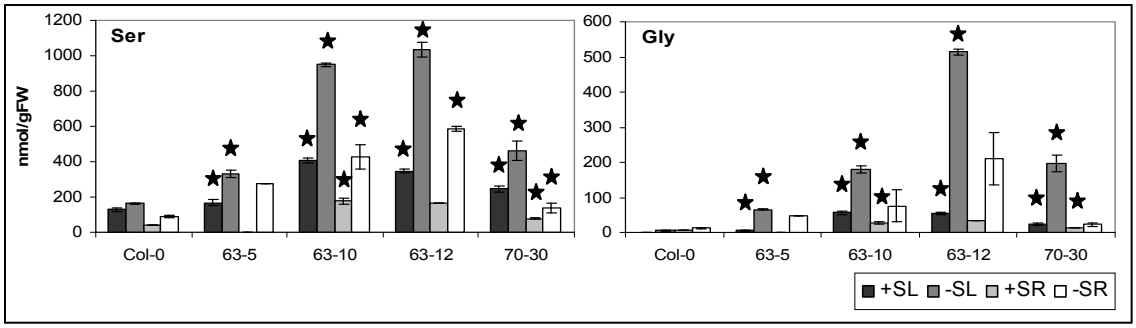


Fig 3-25 Levels of 3-phosphoglycerate derived amino acids in 35S:AP2 plants grown in hydroponics under different sulphur regimes. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different (P < 0,05) in comparison to the respective Col-0 controls. +/-SL, leaf plant material cultivated in control or S-deprived conditions; +/-SR root plant material cultivated in control or S-deprived conditions.

Among the oxaloacetate amino acid family members, the most interesting changes were obtained for cystathionine, methionine and S-methyl-methionine (SMM). In normal sulphur conditions, SMM levels in leaves were 15 times higher in the 63-10 line and 8,8 times higher in the 63-12 line, than in Col-0 leaves (Fig. 3-26). Lines 63-5 and 70-30 did not accumulate SMM amounts at higher levels than wild type. In roots, when comparing to Col-0, the amount of SMM was 4,2, 2,7 and 1,8

times higher in lines: 63-10, 63-12 and 70-30, respectively. Sulphur starvation did not induce any consistent changes in SMM levels in 35S:AP2 plants, in leaves the SMM ratio was close to 1 for starved and non-starved plants, in roots, SMM decreased slightly upon –S, which was the opposite tendency than exhibited by wild type plants.

The direct precursor of SMM, methionine, showed a consistent tendency to accumulate in all tested 35S:AP2 lines (Fig. 3-26). The methionine increase in leaves was 2,2-fold for 63-5 line, 3-fold (63-10), 2,8-fold (63-12) and 1,9-fold for line 70-30, compared to Col-0 plants. In roots, lines 63-10 and 63-12 accumulated metionine over 2-fold higher than wild type. Interestingly, under sulphur limited conditions, methionine levels did not change in Col-0 leaves, while in all 35S:AP2 lines it dropped 3,3 (63-5) to 2 times (70-30) down in leaves, which in result made Met amounts in starved leaves comparable to wild type.

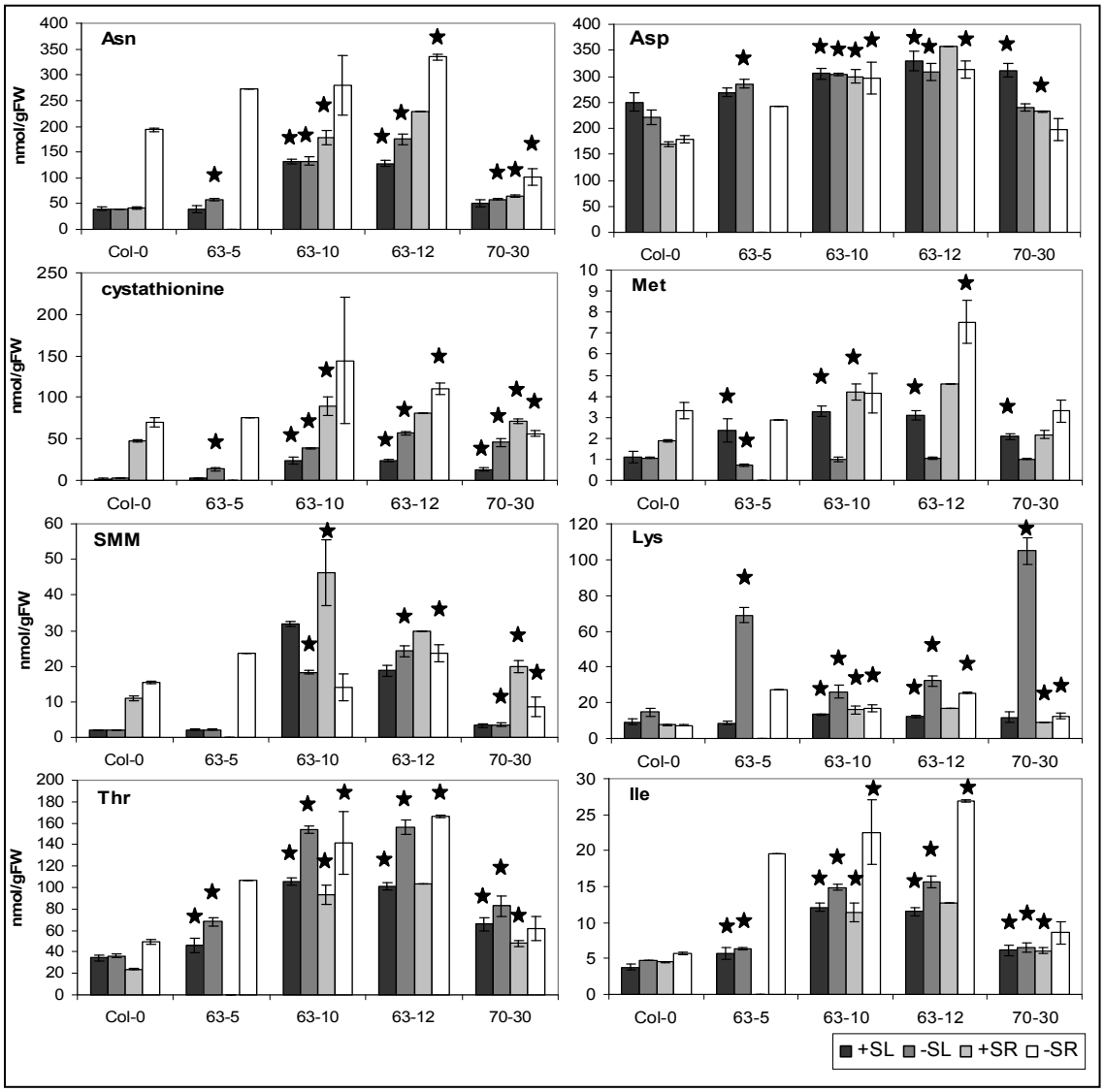


Fig 3-26 Levels of oxaloacetate derived amino acids in 35S:AP2 plants grown in hydroponics under different sulphur regimes. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different (P < 0,05) in comparison to the respective Col-0 controls. +/-SL, leaf plant material cultivated in control or S-deprived conditions; +/-SR root plant material cultivated in control or S-deprived conditions.

The precursor of homocysteine and methionine, cystathionine, which is synthesised in a reaction of O-phosphohomoserine and cysteine, increased over 20 times in the leaves of lines 63-10 and 63-12 and 11 times in 70-30 line, in the 63-5 line – only 1,8 times, compared to Col-0 leaves (Fig. 3-26). Cystathionine levels in roots did not differ significantly from wild type level and did not exceed the 2-fold increase. Opposite to cysteine, homocysteine, and methionine, the cystathionine level increased upon sulphur depletion in leaves of all the lines tested, transgenic and wild type. In Col-0 cystathionine increased 2,3-fold, in 63-5 line – 6,3-fold, in 63-10 – only 1,6-fold, in 63-12 – 2,4-fold and for the 70-30 line the cystathionine increase was 3,5-fold. This resulted in nevertheless higher amounts of cystathionine accumulated in the starved leaves of 35S:AP2 plants, e.g.: 5 times higher in 63-5 line, 14 times higher in 63-10, 21 – in 63-12 and 17 times higher in the line 70-30, than in starved Col-0 leaves.

Levels of asparagine, aspartate, threonine and lysine were remarkably higher in most of the 35S:AP2 lines than in Col-0, for both tissues, but the pattern of their response upon S deprivation was comparable to that of wild type (Fig. 3-26).

All of the members of the α-ketoglutarate amino acid family displayed a significant increase in content in almost all 35S:AP2 lines tested, compared to the wild type (Fig. 3-27). When considering the amino acid levels in leaves of plants grown in normal sulphur conditions, arginine levels increased over 2 times in the 63-10 and 63-12 lines, histidine and glutamine up to 4 times, while the glutamate level increased slightly, only 1,3 times for both of these lines, when compared to Col-0 amino acid levels. In roots, these ratios were even higher, arginine increased 2,6-fold and 3,3-fold in lines 63-10 and 63-12, histidine – 3,8 and 4,5-fold, while glutamate increased in leaves slightly, only up to 1,5-fold, compared to wild type.

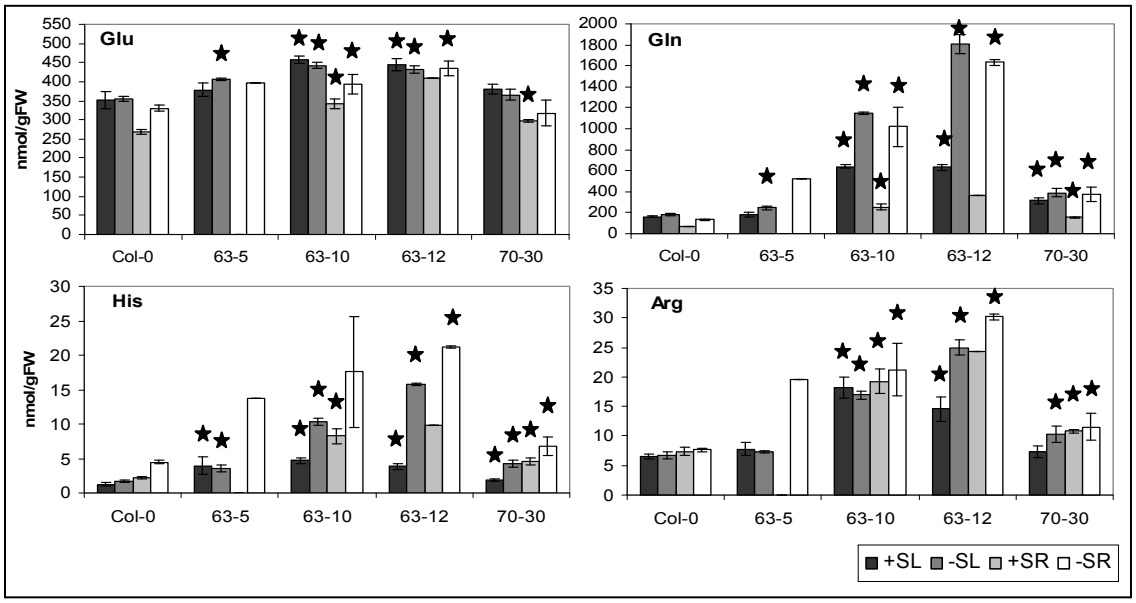


Fig 3-27 Levels of α-ketoglutarate derived amino acids in 35S:AP2 plants grown in hydroponics under different sulphur regimes. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different (P < 0,05) in comparison to the respective Col-0 controls. +/-SL, leaf plant material cultivated in control or S-deprived conditions; +/-SR root plant material cultivated in control or S-deprived conditions.

Asparagine and glutamine, N-rich amino acids, serve as sink compounds accumulating excessive nitrogen upon S limited conditions (Hesse et al., 2004a; Kopriva and Rennenberg, 2004, Nikiforova et al., 2005b). As it was shown, both of them exhibited elevated levels in the lines 63-10 and 63-12 (Fig. 3-26 and Fig. 3-27). This may implicate, that, although the total internal amounts of sulphate in over-expressing lines and Col-0 remained unchanged, elevated transcripts levels of *At2g28550* TF gene may induce the S-starvation sensing by *35S:At2g28550* plants.

Pyruvate-derived amino acids, valine and leucine increased slightly in content, in leaves of *35S:At2g28550* plants (up to 2 times), while alanine levels remained unaffected, when compared to wild type (Fig. 3-28). In roots, alanine (up to 8 times) and leucine (up to 2 times) levels were elevated in *35S:At2g28550* plants, while valine levels remained unchanged. The response pattern of Ala, Ile and Leu under –S was kept comparable to those of Col-0.

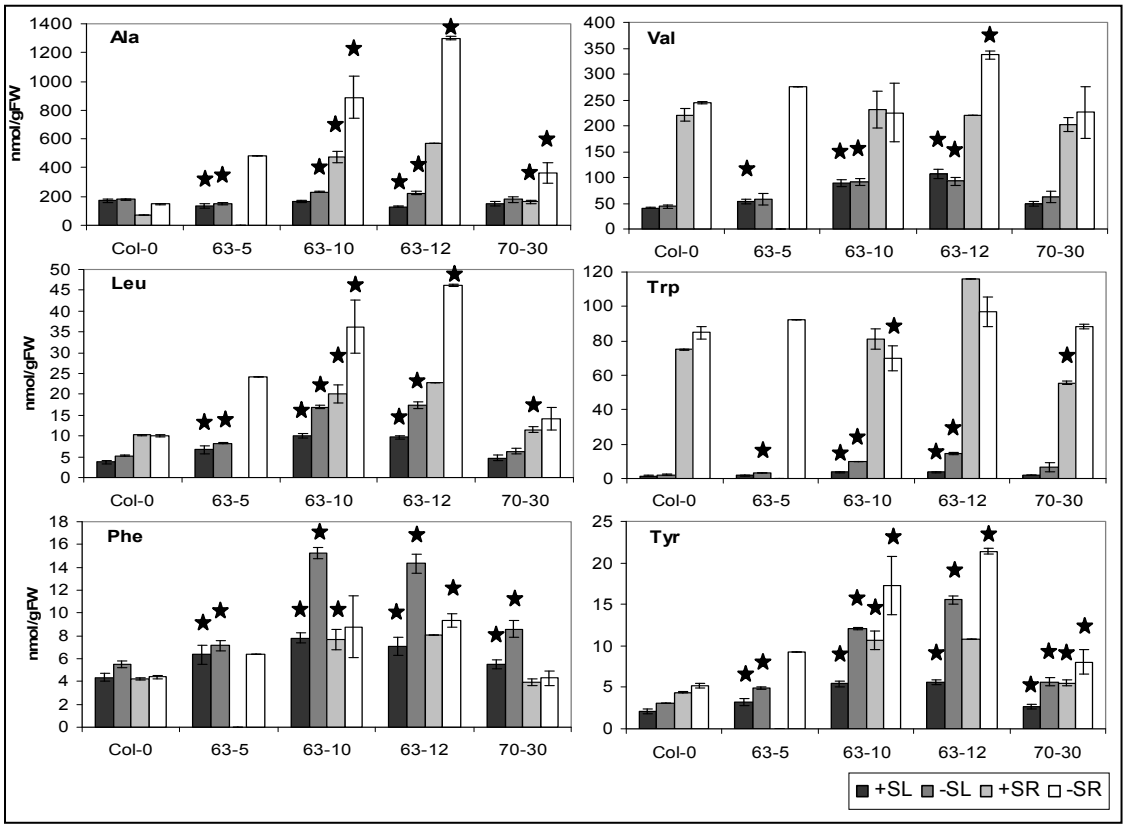


Fig 3-28 Levels of pyruvate and phosphoenolpyruvate derived amino acids in 35S:AP2 plants grown in hydroponics under different sulphur regimes. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different (P < 0,05) in comparison to the respective Col-0 controls. +/-SL, leaf plant material cultivated in control or S-deprived conditions; +/-SR root plant material cultivated in control or S-deprived conditions.

Levels of phosphoenolopyruvate derived amino acids were also affected and exhibited a consistent pattern of alteration in lines 63-10 and 63-12, when compared to Col-0 (Fig. 3-28). In S sufficient conditions they increased up to 2,6 times in leaves. In roots, the highest increase was obtained for tyrosine (2,4 times in 63-10 and 63-12), an increase of phenylalanine did not exceed 2-fold, while tryptophane levels remained unchanged. Sulphur starvation did not induce significant increases of any of these amino acids in wild type plants, while in lines 63-10 and 63-12 these increases were considerably stronger. For tyrosine and tryptophane, the ratio between starved and

non-starved 63-10 and 63-12 plants was in leaves over 2, for phenylalanine it was 2. This led to accumulation of higher amounts of these amino acids under –S, e.g. increased phenylalanine levels are thought to be related to anthocyanins overproduction in 63-10 and 63-12 lines (see chapter 3.B.4.4).

3.B.4.4 Anthocyanins

The phenotype observed for soil grown *35S:At2g28550* plants was displayed also by plants cultivated in hydroponics. Larger rosette leaves and no flower formation were exhibited by lines 63-10 and 63-12 grown in sufficient sulphur conditions. After 10 days of sulphur starvation, these lines exhibited accumulation of anthocyanins and, especially the line 63-10, retardation in growth (Fig. 3-29). Under S limited conditions, an over 40 times overproduction of anthocyanins was found in the lines 63-10 and 63-12, when comparing to starved Col-0 plants (Fig. 3-30).

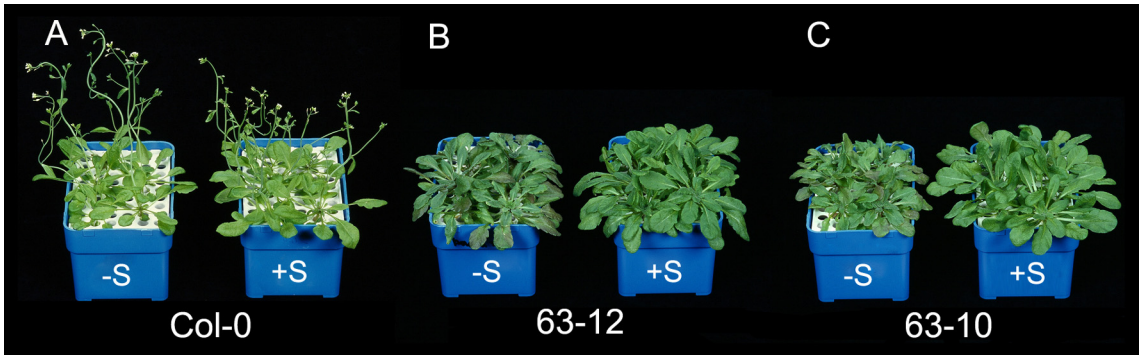
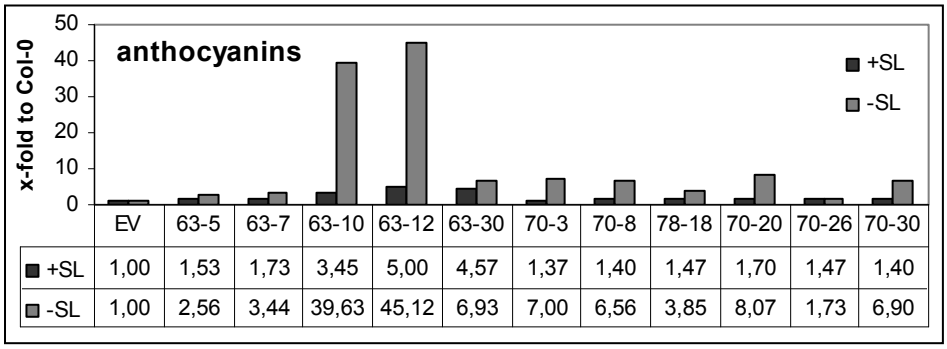


Fig 3-29 Phenotype of selected *35S-At2g28550* lines and Col-0 plants, grown in hydroponics and subjected for 10 days to sulphur starvation.



	EV	63-5	63-7	63-10	63-12	63-30	70-3	70-8	78-18	70-20	70-26	70-30
+SL	1,00	1,53	1,73	3,45	5,00	4,57	1,37	1,40	1,47	1,70	1,47	1,40
-SL	1,00	2,56	3,44	39,63	45,12	6,93	7,00	6,56	3,85	8,07	1,73	6,90

Fig 3-30 Relative level of anthocyanins in 35S:AP2 lines. For each line the average value which is the mean of three independent measurements is divided by the average value for Col-0 plants. +/-SL, leaf plant material cultivated in control or S-deprived conditions.

3.B.4.5 Polyamines

Polyamine measurements revealed remarkable changes in contents in most 35S:AP2 lines, when compared to the wild type levels. In normal S conditions, the soluble fraction of putrescine (fraction 1) increased up to 3 times in leaves of AP2 over-expressing plants, while in roots its amount remained at wild type level (Fig. 3-31). Soluble-conjugated fraction of putrescine (fraction 2) was over

10 times higher in leaves, while the bound fraction (fraction 3) remained unchanged in 35S:AP2 plants, compared to Col-0.

Spermidine levels were in standard S conditions 2 times higher in leaves of 63-10 and 63-12 lines than in Col-0, in roots, only 63-12 line exhibited 2,2-fold increase of this polyamine (Fig. 3-31). Spermine level (in soluble fraction) remained unchanged within all lines tested, besides roots of the 63-12 line, where it increased 2,7 times.

Under limited input of sulphur, the accumulation of putrescine increased, as the conversion to its downstream polyamine, spermidine, is presumably blocked due to reduced SAM (a direct derivative of methionine) availability (Nikiforova at al., 2006; Nikiforova et al. 2005b). In our experimental conditions putrescine accumulates over 3 times in leaves of starved Col-0 plants, while in roots it remained unchanged (Fig. 3-31). 35S:AP2 plants exhibited similar pattern of putrescine changes, which nevertheless resulted in remarkable high putrescine amounts in leaves of 63-10, 63-12 and 70-30 line.

Spermidine (fr1) levels did not change in S limited conditions in leaves of Col-0 plants, while being elevated in normal conditions spermidine levels in 63-10, 63-12 and 70-30 lines exhibited almost 2-fold decrease upon S starvation (Fig. 3-31). Sulphur starvation did not influence the spermine (fr1) levels.

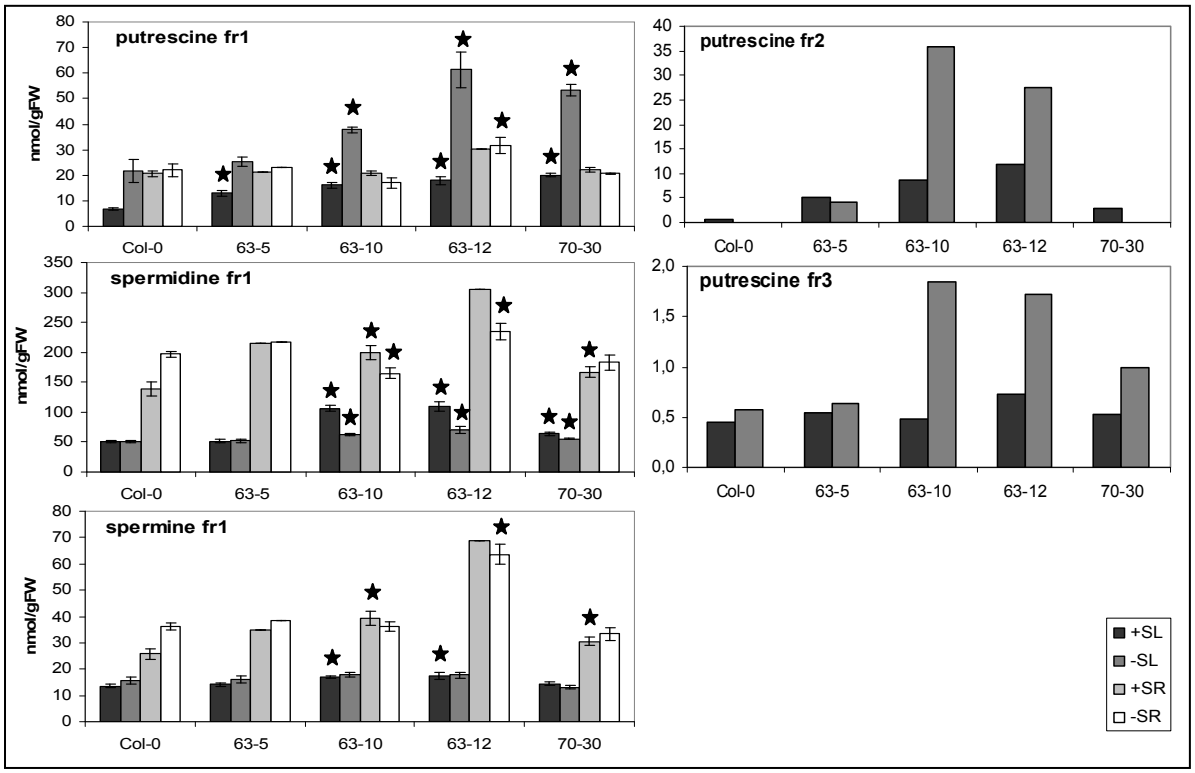


Fig 3-31 Levels of polyamines in 35S:AP2 plants grown in hydroponics under different sulphur regimes. Values ±SD characterise the average of three independent measurements. Asterisks indicate values that are significantly different (P < 0,05) in comparison to the respective Col-0 controls. +/-SL, leaf plant material cultivated in control or S-deprived conditions; +/-SR root plant material cultivated in control or S-deprived conditions.

3.B.5 Molecular characterisation by qRT-PCR

Genes involved in sulphate metabolism and synthesis of other metabolites, which were found to be altered in 35S:AP2 lines (see chapters above), were considered as the most likely biological targets of both investigated AP2 TF genes. Target expression analysis on 35S:AP2 lines using quantitative RT-PCR revealed the increased transcripts levels of many of those genes in roots and down-regulation of their transcription in leaves (Table 3-12, for primer sequences see appendix E).

Among the sulphate transporters, the highest transcript abundancies were found in roots for the low affinity sulphate transporter, SULTR 2;1 (*At5g10180*), especially in the line 63-5 and SULTR 3;2 (*At4g02700*) in lines 63-10 and 63-12, which is not reported to be induced in S starvation. In leaves the strongest down-regulation was detected for the SULTR 3;1 (*At3g51895*), especially for lines 63-10 and 63-12.

5'-adenylylsulphokinase 1 (*APSK1*) was the strongest regulated gene of all the genes acting downstream in the S assimilation pathway. *APSK1* was up to 88 times over-expressed in leaves of line 63-5, strongly over-expressed in lines 63-10 and 63-12 (73 and 55-fold, respectively), and 14 times down-regulated in roots of line 63-10. Interestingly, over-expression of the *At5g60120* gene resulted also in 52-fold up-regulation of *APSK1* in roots of line 70-30. *APSK2* showed the same tendency, although giving lower ratios between 35S:AP2 and Col-0 lines. Two OAS (thiol)lyase genes (*At4g14880* and *At3g61440*) reacted similarly to implemented changes in 35S:AP2 plant transcriptomes, being over-expressed in roots and down-regulated in leaves. All three isoforms of *APS reductase* were down-regulated in leaves, while in roots their over-expression occurred not in all lines tested.

Many genes, which are known to be strongly reacting upon S-deprivation (Nikiforova et al., 2003; Hirai and Saito, 2004), revealed similar induction pattern to those observed for S-assimilation pathway genes. Among them, cation transporter gene from ChaC-like protein family (*At5g26220*) was changed in their expression over 5 times, gene involved in glucosinolates biosynthesis, *CYP79B3* (*At2g22330*) was heavily affected in their expression levels (up to 7-fold) and the gene family of 12-oxophytodienoate reductases (OPR) revealed quite consistant pattern of induction, being strongly inhibited in leaves and induced in roots (Table 3-12).

Table 3-12 Relative expression level of genes involved in primary sulphate assimilation pathway measured for selected 35S:AP2 lines, grown in normal S conditions. Ratios (35S:AP2 lines vs Col-0) higher than 2.0 are depicted in bold, red – up-regulated, black – down-regulated.

AGI code	Gene name	Leaves			Roots			Leaves	Roots
		63-5	63-10	63-12	63-5	63-10	63-12	70-30	70-30
At4g08620	Sultr1;1	NM	NM	NM	**0,17**	0,92	0,63	NM	1,38
At1g78000	Sultr1;2	1,30	0,74	1,31	0,56	1,23	1,14	1,70	1,37
At5g10180	Sultr2;1	0,50	**0,46**	0,53	**16,33**	**2,83**	**6,79**	1,11	**3,18**
At1g77990	Sultr2;2	0,53	**0,23**	0,75	**0,22**	**3,17**	1,85	1,03	1,29
At3g51895	Sultr3;1	**0,45**	**0,13**	**0,17**	NM	NM	NM	1,00	NM
At4g02700	Sultr3;2	NM	NM	NM	1,46	**8,24**	**6,57**	NM	**2,43**
At3g15990	Sultr3;4	0,85	**0,14**	**0,45**	**4,25**	1,66	1,49	**2,61**	1,47
At5g13550	Sultr4;1	1,28	**0,42**	0,73	**2,60**	**2,44**	**2,48**	1,30	**2,16**
At3g12520	Sultr4;2	**0,47**	**0,24**	0,60	1,53	1,15	1,25	1,13	1,32
At1g80310	Sultr5;1	**0,41**	**0,29**	0,65	0,54	1,34	1,00	0,83	0,87
At3g22890	ATPS1	0,60	**0,08**	0,76	**3,62**	**2,64**	**2,48**	0,96	1,92
At1g19920	ATPS2	**0,23**	**0,25**	0,72	**3,95**	**8,09**	**6,54**	0,77	**4,58**
At4g14680	ATPS3	0,56	**0,09**	**0,25**	**11,48**	0,71	0,77	0,91	1,28
At5g43780	ATPS4	0,70	**0,28**	0,74	0,56	1,37	1,06	1,09	0,98
At2g14750	APSK1	0,74	**0,07**	**0,33**	**88,43**	**73,09**	**55,91**	1,05	**52,11**
At4g39940	APSK2	0,94	**0,10**	**0,38**	**18,43**	**5,70**	**7,00**	1,30	**5,22**
At4g04610	APR1	**0,33**	**0,21**	**0,19**	**7,13**	1,45	**2,85**	0,85	**2,95**
At1g62180	APR2	**0,29**	**0,13**	**0,40**	1,93	1,20	1,58	0,66	**2,34**
At4g21990	APR3	**0,17**	**0,14**	**0,22**	**3,96**	**0,35**	1,04	1,03	1,62
At1g55920	SAT 1	0,51	**0,39**	**0,27**	**2,77**	0,59	1,11	0,58	0,79
At3g13110	SAT A	1,24	**0,16**	0,66	**2,76**	**2,30**	**2,44**	1,37	1,65
At5g56760	SAT 52	1,11	**0,22**	**0,40**	1,67	**2,46**	**2,36**	1,74	**2,02**
At2g17640	SAT2 106	**0,23**	**0,16**	**0,46**	0,69	1,29	0,88	0,52	1,19
At4g14880	1OASTLA1	**0,18**	**0,22**	**0,31**	**16,90**	**22,63**	**30,58**	1,23	**18,50**
At2g43750	2OASTLB	**0,31**	**0,46**	0,75	1,11	**2,05**	**2,04**	0,85	1,31
At3g04940	5OASTLD1	**0,24**	**0,34**	0,50	1,03	**2,35**	**2,21**	0,63	1,48
At3g22460	6OASTL	**0,29**	**0,10**	**0,30**	0,89	1,22	1,08	0,63	1,52
At3g61440	7OASTLC1	**0,38**	**0,34**	0,72	**12,56**	**11,91**	**11,41**	0,74	**7,46**
At5g24660	putative protein (vikin-like1)	**0,36**	**0,23**	1,10	1,75	0,68	1,10	0,68	1,79
At1g75280	IFR	**0,31**	**0,25**	**0,38**	0,52	0,68	NM	0,86	0,98
At3g05400	sugar transporter, putative	**0,32**	**0,18**	0,90	0,50	1,07	0,90	0,89	0,72
At5g26220	ChaC-like protein family of cation transp.	**0,21**	**0,11**	**0,27**	**5,24**	**2,00**	**4,78**	**0,23**	**5,07**
At1g36370	Ser hydroxymethyltransferase (SHMT)	**0,36**	**0,10**	0,51	0,99	1,42	1,09	0,72	1,56
At3g44320	NIT3	0,80	**0,46**	0,86	0,64	**2,09**	1,64	1,29	**2,42**
AT1G18870	isochorismate synthase, putative	0,80	**0,14**	0,38	1,97	**4,85**	**3,05**	0,87	**2,98**
At1g08830	superoxide dysmutase (CSD1)	**0,33**	**0,13**	**0,26**	**2,01**	**0,24**	0,92	**0,10**	**0,18**
At1g76680	OPR1	0,52	**0,12**	**0,46**	**4,20**	0,69	1,19	1,16	1,96
At1g76690	OPR2	**0,37**	**0,07**	0,63	**7,86**	**2,66**	**2,84**	0,96	**2,99**
At2g06050	OPR3	0,71	**0,06**	**0,09**	**5,10**	1,79	**2,31**	1,51	1,43
At1g75270	DHAR	**0,33**	**0,20**	**0,49**	1,63	**2,16**	1,82	0,75	1,64
At2g22330	putative cytochrome p450 (CYP79B3)	0,89	**0,30**	**0,35**	**7,93**	**2,25**	**3,60**	**2,01**	**2,53**

3.B.6 Searching for regulated elements – network analysis of *TOE1* and *TOE2* AP2 transcription factor genes

The systematic accumulation of gene expression and metabolic data allowed to reconstruct a dynamic network of informational flows in *Arabidopsis* plants perturbed by sulphur depletion. Basic underlying dataset for the response network compiles S-starvation transcript and metabolic profiles data from the experiment presented in this work (section 3A) and experiments performed before (Nikiforova et al., 2003), and does not regard the replenishment studies. Network reconstruction was performed as described before (Nikiforova et al., 2005a). Assuming that the depletion of sulphate from the medium is the primary cause of the system excitement, a general 'cause-to-effect' directionality of information fluxes along network paths from sulphur to distant elements has been implemented. The data from 16 conditions (time series of S starvation and corresponding controls) for 2014 entries which passed selection criteria (see chapter 2.5.3) were used for the network reconstruction presented here.

The response network of informational flows in S-depleted *Arabidopsis* plants was used to search for elements possibly regulated by two AP2 TF genes, *TOE1* (*At2g28550*) and *TOE2* (*At5g60120*). Both AP2 genes passed the selection criteria and were present among the 2014 entries used for the network reconstruction. After implementing the casual directionality, the *TOE1* gene located in the class 6, while *TOE2* was even more distant from sulphur (class 0), being located in the class 7 (Fig. 3-32).

Although *TOE1* was not found to be tightly connected to sulphur, it appeared to be a hub for 85 direct neighbours (Fig. 3-32; Appendix D). 29 vertices were located upstream (class 5) to the *TOE1* gene, 53 belonged to the class 6, while only 3 entries represented the downstream class 7. Of 85 neighbours, 8 were transcription factor genes, each of which belonged to a different TF family, such as: C2C2-Gata family, C2C2-CO-like family, bHLH, WRKY, NLP, HMG and Homeobox (HD-ZipII) family, while ETR1 TF gene (*At1g66340*) is known to be an ethylene-response protein and takes part in ethylene signal transduction. Three of these TF genes (ETR1, GATA3 and the NLP-family gene) were located upstream to the *TOE1* gene (in the 5th class in the network), while five others belonged to the class 6. Among other close neighbours of the *TOE1*, two were involved in threonine degradation: aminoacetone oxidase (*At3g43670*), producing methylglyoxal, and involved in the next step of this pathway, lactoylgluthathione lyase (*At1g15380*), which revealed strong down-regulation under S limited conditions. Two 5th class neighbours were involved in a protein degradation: AtFBL5 (*At1g77000*) and serine carboxypeptidase II-like protein (*At5g23210*). Chorismate mutase (*At1g69370*), involved in phenylalanine and tyrosine biosynthesis, and strongly decreased in expression fructose-bisphosphate aldolase (*At4g26530*), which catalyses the degradation of sucrose to ethanol and lactate (in glycolysis or gluconeogenesis processes), were both located in the 5th class of the response network. Asparagine synthetase (ASN3, *At5g10240*) and glutamine amidotransferase/cyclase (*At4g26900*), involved in the histidine biosynthesis, were found to be located in the class 6 of the direct *TOE1* neighbours. Interestingly, a disulphide oxidoreductase (*At1g62560*), catalysing interchanges between reduced proteins with SH groups and proteins with oxidised

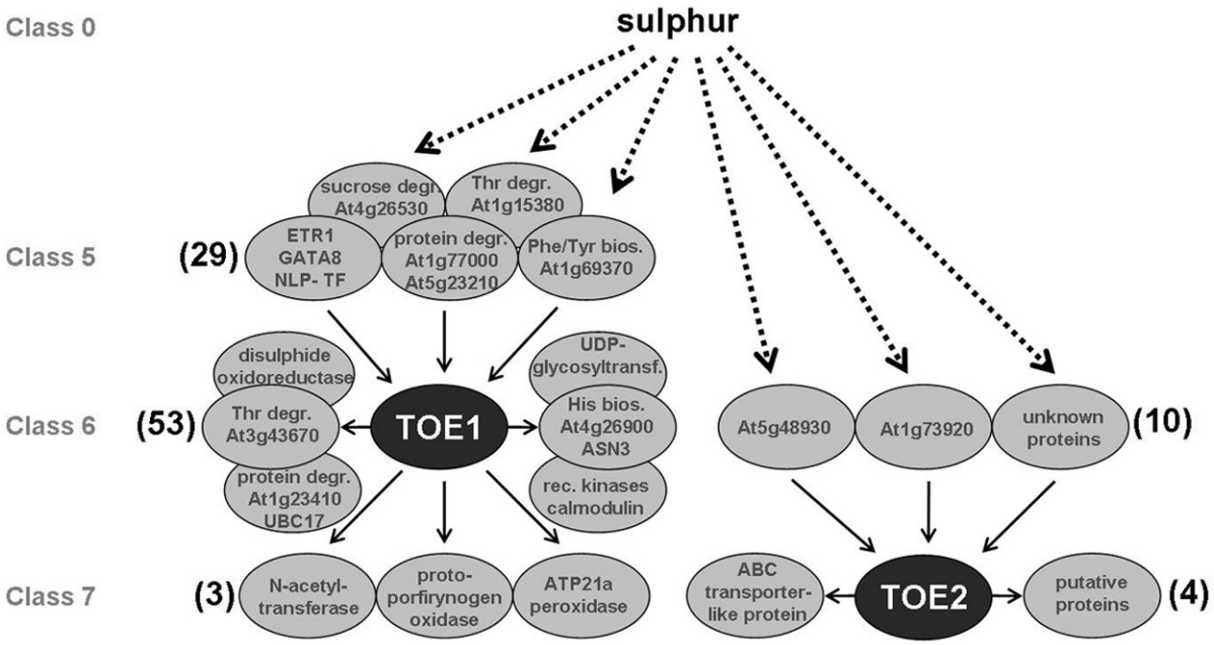


Fig 3-32 Scheme of interactions of TOE1 and TOE2 genes in the reconstructed gene-metabolite response network, based on *Pajek* visualisation. Gene and metabolite vertices are depicted as ovals. The AP2 TF TOE1 (At2g28550) belongs to the class 6, the TOE2 (At5g60120) belongs to the class 7 of reconstructed network. Noteworthy examples of their direct neighbours are displayed as gray ovals. Figures in brackets show the total number of direct neighbours falling into respective class category. Solid arrows indicate interactions among direct neighbours, dashed arrows indicate influences of the sulphate depletion assumed to be the primary cause of the system excitement.

disulphide bonds, was strongly reduced in expression in S-limited conditions and located among the 6th class of *TOE1* neighbours. In contrast to that, 6th class UDP-glycosyltransferase, taking part in a number of reactions in the cytokinins biosynthesis pathway, was strongly induced under S-starvation. Remarkably, among class 6 *TOE1* neighbours, two genes involved in protein degradation (E2 ubiquitin-conjugating enzyme 17 and ubiquitin extension protein) were found, as well as three genes taking part in protein postranslational modifications, two receptor kinases and calmodulin involved in calcium signalling. Only three genes were located downstream to the *TOE1*: N-acetyltransferase (*At5g13780*), protoporphirinogen oxidase, involved in proto- and siroheme biosynthesis, and ATP21a peroxidase localised in endomembrane system and responsive to oxidative stress.

Unlike *TOE1*, the *TOE2* TF gene was located in the class 7 of the response network and formed a hub of only 14 genes (Fig. 3-32; Appendix D). Ten of them belonged to the upstream class 6, among which genes such as anthranitrilate N-benzoyltransferase (*At5g48930*, involved in metabolism of phenylpropanoids) and a putative lipase (*At1g73920*, catalysing a triacylglycerole degradation) were found. Of four direct *TOE2* neighbours, the function of one is known. The *At5g58270* gene is coding a mitochondrial ABC transporter involved in heavy metal resistance, especially to cadmium ions. None of the *TOE2* neighbours located in the downstream class 8 and none of them was a TF gene.

Location of the *TOE1* and *TOE2* genes in the response network and the variety of function represented by their closest neighbours suggest that, if the S-starvation response is regulated at the transcriptional level, *TOE1* and *TOE2* genes are not directly involved in the first line of defence against the sulphate deficiency.

4 DISCUSSION

4.A Identification of sulphate-regulated transcription factor genes

4.A.1 Why was there a demand for a new approach to study sulphur deficiency?

During the past decade, remarkable progress has been made in the basic understanding of regulatory mechanisms, genes and proteins involved in sulphur assimilation. Studies with different model organisms, such as *Escherichia coli* (Phillips et al., 1989; Marincs et al., 2006; LaMonte and Hughes, 2006; Augustus et al., 2006), *Saccharomyces cerevisiae* (Cherest et al., 1969; 1985; Thomas et al., 1990; 1992a; 1992b; Kuras et al., 1996; 1997; Thomas and Surdin-Kerjan, 1997), *Neurospora crassa* (Fu et al., 1989; Paietta, 1990; 1992; 1995; Marzluf, 1997; Sizemore and Paietta, 2002), *Aspergillus nidulans* (Mountain et al., 1993; Piotrowska et al., 2000; Paszewski et al., 2000; Natorff et al., 2003) and *Chlamydomonas reinhardtii* (de Hostos et al., 1988; Yildiz et al., 1994; 1996; Davies et al., 1994, 1996; Ravina et al., 1999; 2002; Pollock et al. 2005), contributed to our understanding of sulphur assimilation regulatory processes. Moreover, these studies elucidated that the assimilatory pathway of plants is principally identical to the characterized pathways of these organisms. Nevertheless, many questions about the transcriptional regulation of sulphate assimilation in plants remain unanswered, mostly because orthologous genes of the corresponding regulatory factors have not been found. Thus, it was not possible to transfer the knowledge about transcriptional regulatory schemes from mentioned above model organisms directly to higher vascular plants, so the plants signaling pathway of sulphur nutritional stress remains largely unexplored (Saito, 2004).

With the genome sequence of *Arabidopsis* and rice now available, plant science has stepped forward into a new phase. Post-genomics studies such as transcriptomics, proteomics, and metabolomics will bring about a breakthrough for the functional elucidation of genes and for an understanding of a whole process of living cells (Hirai and Saito, 2004). Concerning studies of sulphur metabolism, several reports have recently been published describing the transcript profiles of S-starved *Arabidopsis*.

In the publications describing the transcriptome under –S (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003), *Arabidopsis* plants of ecotype Columbia were grown on sterile agarose-solidified media in plates. Wild-type plants were grown under continuous –S conditions, or the plants grown under +S control condition were transferred to –S or OAS-supplemented media. Maruyama-Nakashita et al. (2003) also used *sel1-10*, a knockout mutant of Sultr1;2. Leaves, roots or whole seedlings (Nikiforova et al., 2003) were analysed for transcript profiles by DNA macro- or microarray, however, only around 8000 genes (Maruyama-Nakashita et al., 2003;

Nikiforova et al., 2003) or 9000 genes (Hirai et al., 2003) were analysed in these studies. Detailed comparison of data from array experiments and metabolic profiles are done by Hirai and Saito (2004) and Nikiforova et al. (2004, 2005b). By combining these results, networks of several pathways under –S were clearly drawn, in addition to the S uptake and the assimilation pathways as expected.

However, no identical transcription factor is identified as common in more than two studies (Hirai and Saito, 2004). One reason is that each array used in these studies contained only approximately one-third of all *Arabidopsis* genes. The expression levels of transcription factors are expected to be low and the ESTs of the transcription factor were not always available for the preparation of the array. Alternatively, in these studies, final physiological responses varied depending on the severeness of S shortage, the plant-growth stage, and the period of –S stress; that is, the most downstream genes in the signal transduction pathway were different from experiment to experiment. Hence it is reasonable that different transcriptional factors were regulated in these experiments.

It should also be taken into consideration that often, the abundance of transcripts for specific regulators does not change under conditions in which the regulator functions to alter gene expression, as suggested by Davies et al. (1999). Indeed, glucose-deprived yeast cells and sulphur-deprived *Chlamydomonas* do not exhibit altered levels of the *SNF1* (Celenza and Carlson, 1984) and *Sac3* (Davies et al.,1999) transcripts, respectively. Also the recently identified SLIM1/EIL3 transcription factor, which controls both the activation of sulphate acquisition and degradation of glucosinolates under –S conditions, was reported to be not regulated itself by these conditions at the transcriptional level (Maruyama-Nakashita et al., 2006). SLIM1/EIL3, however, has been identified in a genetic approach, in which a series of *Arabidopsis* mutants with a sulphur limitation-responsive promoter-GFP reporter system were screened for a sulphur limitation response-less phenotypes. This clearly shows, that approaches analyzing changes in transcription target exclusively differentially expressed regulators and, thus, different approaches are suitable for identification of differentially and non-differentially expressed candidates.

Insufficient analysis of transcription factors genes involved in sulphate metabolism performed so far created high demand for its more expanded investigation. Hence, we aimed at identifying transcription factors that control sulphate metabolism in *Arabidopsis thaliana*. We targeted the differentially expressed regulators, as we postulated that factors regulating *Arabidopsis* responses to inorganic sulphate deficiency change their transcriptional levels under sulphur-limited conditions.

4.A.2 Transcript levels for 1417 *Arabidopsis* TF genes were measured with high specificity and precision – advantages from using qRT-PCR resource

In the short term S-starvation approach, a unique public resource for studying the expression of TF genes in *Arabidopsis* (Czechowski et al., 2004), was used to identify TF genes potentially involved in sulphate signalling. This resource, which is based upon highly multiplexed quantitative RT-PCR with gene-specific primers, enabled us to measure transcript levels in seedlings for 1417 TF genes with high specificity and precision. Transcript levels for *Arabidopsis* TF genes, represented by $(1+E)^{-\Delta CT}$, varied over 6 orders of magnitude (Fig. 3-8). The highest TF expression level was close to

that of the house keeping genes (*UBQ-10* and *ACT-2*) and the lowest just on the limit of detection, which was established to be of 1 transcript per 1000 cells (Czechowski et al, 2004). Such a range in TF gene expression level detection has not been reported for plants by any hybridisation-based technologies. Presumably, this great range reflects not only differences in the expression level of different TF genes within any one cell-type, but also differences between cells of different tissues and organs.

As a part of a wider screen to identify candidate genes regulated by changes in sulphate availability we performed expression profiling using Affymetrix ATH1 array, using the same RNA samples as for qRT-PCR (1st experiment) and RNA from the biological replica (2nd experiment). Transcript levels of 22750 genes for two independent experiments were estimated. Comparison of the data on TF transcript abundance obtained by qRT-PCR with those obtained for the same RNA samples using Affymetrix chips yielded some interesting observations.

The range of values obtained with qRT-PCR was two orders of magnitude greater than that obtained with Affymetrix chips (10^5 vs. 10^3, respectively). As shown by Czechowski et al. (2004), qRT-PCR yields a constant ΔC_T for each X-fold change in initial DNA concentration over the whole range of detectable DNA concentrations. This is not true for DNA array-based methods, which suffer from an exponential decrease in signal intensity as transcript levels fall, due to second order kinetics of hybridisation (Holland, 2002). This could account for the narrower range of values obtained with Affymetrix chips compared to qRT-PCR (Fig. 3-10).

A large majority (94,5%) of the 456 genes that were categorised as ‘absent’ by Affymetrix software was detected by real-time PCR, albeit at lower levels than other TF genes, as expected. Thus, the qRT-PCR appeared to be the more sensitive method than the ATH1 chip hybridisation and therefore more relevant especially for usually low expressed transcription factor genes.

Quantitative accuracy of qRT-PCR and Affymetrix chips was also compared. A plot of the absolute signals for 1236 TF genes given by the two methods revealed a rather weak correlation in the range corresponding to highly expressed genes and no correlation for genes expressed at lower levels (Fig. 3-10). Unlike quantitative RT-PCR, hybridisation-based technologies like Affymetrix chips are qualitative and there is not a strict linear relationship between signal strength and transcript amount for different genes.

The qRT-PCR resource is very flexible tool – it is possible to add, remove, or replace primer pairs at any time. A small platform for expression profiling of genes from sulphate uptake and assimilation pathway was established as part of this project. Using previously described criteria (chapter 2.3.3.2), primer pairs covering most sulphate transporter genes and genes of enzymes taking part in S-assimilation, were designed and aliquots were arrayed on 384-well plate (for primer sequences see Appendix E). By using the Evolution P3 liquid handling system, (Perkin Elmer; collaboration with System Regulation Group, MPI-MP Golm, Germany), set-up qRT-PCR reactions was fully robotized. One 384-well plate containing four sets of S-assimilation gene primers could serve for measuring four different cDNA samples. Thus, it is possible to measure the expression of S-assimilation genes for many samples in a multiparallel approach.

The platform of sulphate assimilation genes was used prior to the entire TF-library screening by quantitative RT-PCR to ensure that the plant material was induced by sulphate deprivation and replenishment (chapter 3.A.2.5). Another advantage from qRT-PCR S-assimilation genes platform was taken for expression analysis of T-DNA insertion mutants (chapter 3.A.5.6) and 35S:AP2 overexpressing plants (chapter 3.B.5). These lines were expected to exhibit changes in expression of genes connected to sulphate metabolism, so the possibility to measure only this subset of genes instead of processing whole-genome chips allowed to adjust effort and costs.

4.A.3 Molecular and physiological responses to sulphur deprivation and replenishment revealed a systemic internal rebalancing of plant metabolism

To clarify the early changes in the transcriptome in response to sulphur deficiency, *Arabidopsis* seedlings were subjected to relatively short-term sulphur deficiency. Axenicly grown 10-days old seedlings were transferred to sulphate-free conditions for 48 hours. However, under these conditions, *Arabidopsis* seedlings exhibited no typical phenology of S-limited plants, which are known to be: reduced chlorophyll, accumulation of anthocyanins in the leaves, and pronounced root and especially lateral root growth (Nikiforova et al., 2003). In contrast to that, *Arabidopsis* seedlings grown axenicly in the same conditions, but deprived for nitrate or phosphate, showed typical, phenotypic response to nitrogen or phosphate deprivation, respectively (Scheible et al., 2004, Morcuende et al., 2007). Although of key importance in the life of plants, sulphur is a relatively minor component in comparison to nitrogen. For example, the abundance of sulphur is about 7% that of nitrogen in shoot tissues (*Biochemistry and Molecular Biology of Plants*, 2000). Hence, the observed differences in starvation symptoms become reasonable. Presumably, since the starvation yielded no changes in phenotype, also the re-supply of sulphate to S-deprived plants had no observable effect on plant phenotype.

At the molecular level, deprivation of sulphate leads to increases in the expression of sulphate-responsive genes, as reviewed by Hirai and Saito (2004) and Nikiforova et al. (2004, 2005b). We analysed our expressional dataset for reliability by comparison with results on sulphur starvation experiments made earlier by a number of research groups, where the behaviour of individual S-responsive genes was described (Nikforova et al., 2003; Maruyama-Nakashita et al., 2003; Hirai et al., 2003; Hirai and Saito, 2004). Among plant S-assimilation genes, known to be induced by S-deprivation, the expression of genes encoding two high affinity sulphate transporter genes SULTR 1;1, SULTR 1;2, one low affinity sulphate transporter SULTR 2;1, and isoformes of APS reductase, was found to be induced also in our experiment (Table 3-1). This subset of genes was used in this project as molecular markers of S-deprivation. Among other genes, which were reported before to be sulphate-inducible, transcript analysis done in this work revealed strong induction of two isoforms of putative isoflavonoid reductases (*IFR*; *At1g75280* and *At1g75280*), carbonic anhydrase (*At1g23730*), 12-oxophytpdieonoate (*OPR2*; *At1g76690*), lipoxygenase (*AtLOX2*; *At3g45140*), nitrilase 3 (*At3g44320*), *etc.* (Table 3-4). Moreover, of 24 of S-responsive genes identified in our experiment, 11

(46%) contained a conserved *cis*-acting element (named SURE) in the upstream region of their sequences (Table 3-4; Maruyama-Nakashita et al., 2005).

Genome-wide studies done in this work allowed to monitor the expressional changes for whole biosynthetic pathways, as it was demonstrated for genes from biosynthesis pathway of glucosinolates (Table 3-5) and genes responsible for amino acids biosynthesis (Table 3-6). Whole-genome chips were not reported to be used for expressional studies of sulphate deprivation to date, so using ATH1 chips in our approach was a great contribution to that research field. This revealed involvement of many new genes in plant systematic response against sulphate limitation (Table 3-2) and allowed to demonstrate simultaneously all obtained results by the data visualization tool *MapMan* (Fig 3-7).

When mapping the transcript and metabolite data on the known amino acid biosynthesis pathways (*Biochemistry and Molecular Biology of Plants*, 2000), a consistent picture of responses starts to emerge (Fig. 4-1). Despite the distinct challenge of the system by deprivation of sulphate we observe a rather complex response with cross-effects on other pathways. Some of the results are obviously consistent with the expectations or corroborate previous findings, such as SAT induction and serine/glycine accumulation coupled to serine hydroxymethyltransferase (SHMT) induction. Further, S-starvation resulting in SAM depletion (Nikiforova et al., 2006) induces genes of SAM synthesis and recycling to reconvert the demethylated SAM back to methionine and, eventually, SAM. However, there are also significant differences between results obtained in previous and this study.

As expected, ion measurements revealed strong significant decreases of sulphate after 2 days of sulphur starvation (Fig. 3-2). Starved seedlings contained only 4% and 6% (for the experiment 1 and 2, respectively) of the sulphate level measured in control plants grown on full nutrition medium. Chemical element analysis revealed almost 80% decrease of the internal sulphur level in S-starved seedlings (Fig 3-3). The reduced sulphate availability caused a block for cysteine synthesis as insufficient amounts of sulphide were provided through the uptake and sulphate reduction pathway. As expected, in *Arabidopsis* seedlings, in two independent experiments performed, levels of all four thiol-compounds decreased significantly after 2 days of sulphur starvation, when comparing to the plants grown on sulphur-sufficient medium (Fig. 3-1), which is consistent with observations reported previously (Nikiforova et al., 2003; Hirai et al., 2003).

As it is known from previous studies (Nikiforova et al., 2003; Nikiforova et al., 2006), with a limited input of sulphur, levels of sulphur-containing metabolites such as cysteine and gluthatione decrease, while the precursor, serine, accumulates, which was detected also here (Fig. 3-4). The changes of glycine levels reflect the levels of serine.

Despite the fact that cysteine serves as precursor of methionine through a transsulphuration reaction (Hesse and Hoefgen, 2003; Hesse et al., 2004), methionine levels were not grossly affected by the limited input of sulphur (Fig. 3-4).

However, the relationship between levels of cysteine and methionine in S-starved seedlings is worth considering. Nikiforova et al. (2003; 2004 and 2006) reported cysteine levels to be decreased to immeasurable amounts under –S, while levels of methionine did not exhibit significant changes neither before visible starvation symptoms, nor after. This phenomena is discussed as a proof for a indispensability of methionine. Although the strong decreases of cysteine are a great expense for

plants, the maintenance of sufficient methionine levels, is suggested to be of a high priority. The this metabolite as has been shown in various studies (Nikiforova et al., 2003; 2004; 2006). Presumably, the level of methionine is often kept constant despite the sulphur starvation, as methionine is the precursor for S-adenosylmethionine (SAM), the second most widely used enzyme substrate after ATP (Cantoni, 1975) and the methyl group donor for almost all biological methylation reactions (Droux et al., 2000).

In this study, both cysteine and methionine decreased. Cysteine decreased on average 3-fold (Fig 3-1), while methionine decreased 1,2 and 1,8-fold (for the experiment 1 and 2, respectively) in S-depleted seedlings, compared to control (Fig 3-4). Why the short sulphur starvation (48 hours), applied in this study, resulted in misbalances of methionine levels, and not the longer one studied before (Nikiforova et al., 2003)? We think, that the pre-cultivation of Arabidopsis seedlings in the 150 µM sulphate medium might have played a large role (see chapter 2.2.3). The 150µM sulphate allowed seedlings to germinate and grow, however, after 7 days of cultivation, the sulphate in that medium was consumed by plants and decreased to the immeasurable amounts. This forced plants to mobilise their sulphate resources. The following S-starvation applied for next two days presumably appeared to be a more severe stress for these seedlings, than it would be without the pre-cultivation. Pre-dispose of plant sulphate resources made them more sensitive to following SO_4^{2-} limitations and caused misbalances of methionine levels.

It is also possible that reduced protein synthesis or even protein breakdown, which was observed in previous studies (Nikiforova et al., 2003; 2005b) under conditions of sulfate starvation (long term starvation of mature plants in the 100 µM sulphate medium), might help to keep methionine levels constant through reducing the loss of methionine into proteins to keep C1-metabolism functional. In our experimental conditions (pre-cultivation in the 150 µM sulphate medium, rapid transfer of pre-cultivated seedlings into zero sulphate medium), plants had no chance for a restraint/suppression of the whole metabolism when subjected for the S limitations stress. Moreover, rapid growth of young seedlings presumably created a high demand for protein biosynthesis and even increased the loss of methionine into proteins. Thus, due to our special experimental design, we were able to break the plant tendency to keep methionine levels constant and induced the significant decreases in methionine levels. This had several consequences manifested mainly in changes in the aspartate family metabolites.

In consequence to the reduced methionine content and presumably reduced SAM content the highest accumulation was detected for putrescine, as the conversion to its downstream polyamine, spermidine, was presumably blocked (Nikiforova et al., 2006; Nikiforova et al. 2005b). Putrescine content remained elevated even after re-supply with sulphate.

Levels of homoserine, threonine and isoleucine also differ between experiments. Nikiforova et al. (2003; 2005b; 2006) reported their accumulation under S limited conditions which was discussed as a proof for the critical regulation of threonine/methionine homeostasis through the CgS/TS branch point (Kreft et al., 2003). Here, however, only a slight accumulation of threonine is observed, while isoleucine level remain unchanged. Astonishingly, the level of homoserine decreased, which might be

explained by its high demand for downstream metabolisation to methionine, which in these conditions is also decreased (see above).

On the other hand, only when the branch point enzymes are altered, the plant responds with alterations in methionine levels as shown through the analysis of mutants altered in CgS or TS activity or transgenic approaches altering CgS and TS expression and hence enzyme abundance (Chiba et al., 1999, 2003; Kreft et al., 2003; Bartlem et al., 2000). Why in our experimental conditions decreased levels of methionine are not accompanied by increased expression of CgS, remains unknown (Table 3-6). Additionally, it was show that manipulation of downstream genes of methionine synthesis, CbL and MS, has no effect on methionine levels (Zeh et al., 2001, 2002; Maimann et al., 2000, 2001). Astonishingly, upon S-starvation applied in this work, expression of two genes encoding CbL (*At4g23600* and *At1g64660*) were found to be highly overexpressed (Table 3-6).

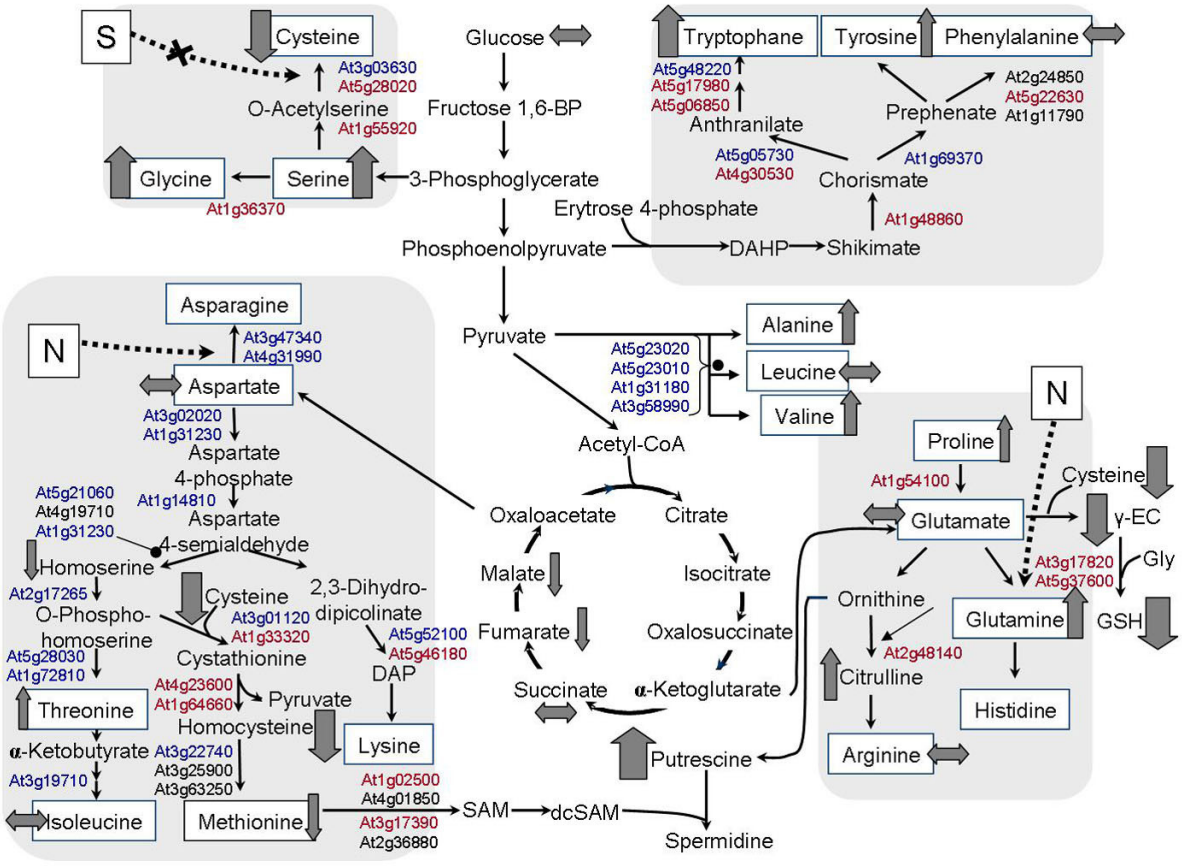


Fig 4-1 Superposition of changes in metabolite pools and gene expression on the amino acid biosynthetic pathways and the main anaplerotic reactions providing the carbon backbone precursors of plant amino acids of Arabidopsis thaliana seedlings exposed to sulphur deficiency. The amino acids are grouped according to the biosynthetic families (*Biochemistry and Molecular Biology of Plants*, 2000). Average values of two independent experiments from this study have been calculated. Grey arrows indicate changes in metabolites, thickness of the arrow provides an indication on the strength of the observed changes and direction of the arrow indicates induction (up) , reduction (down) or no alteration (horizontal) of the respective metabolite. Gene names are provided as the AGI codes following *AraCyc* nomenclature (TAIR; www.arabidopsis.org). Colour of the AGI code indicates change of the respective gene expression: red (up-regulation), blue (down-regulation), black (no change).

The pyruvate-derived amino acids, alanine and valine, were increased in this approach. No alteration in gene expression, though, of the branched chain amino acids was detected, besides decreases of the genes of the leucine biosynthetic branch. We thus must assume rather a regulation

at the enzyme activity and control level. Direct amination of pyruvate leads to alanine which might thus provide another additional N dump and explain the accumulation of alanine.

Other responses affecting amino acid composition are not immediately conclusive. Among the aromatic amino acids tryptophan and tyrosine show clearly elevated levels. Some of the genes of tryptophan biosynthesis are induced supporting the metabolite data. We postulate that accumulation of aromatic compounds is triggered to compensate for the loss of the scavenging system of reactive oxygen species (GSH/GSSG) and its substitution by alternatively synthesized phenolic compounds. Interestingly, while serine cannot be converted to cysteine and eventually glutathione any longer, it might immediately serve as precursor in tryptophan biosynthesis from indole and serine. Notably, among the most highly induced genes all studies identified a putative isoflavonoid reductase homologue (*At1g75280*) (Nikiforova et al., 2003; Hirai et al., 2003), which is the case also in this study.

One major problem for plants exposed to sulphate starvation results from the necessity to deal with the relative excess of nitrate and ammonium, which is derived as well from protein and amino acid degradation as from uptake and assimilation. The imbalance of ‘‘normal’’ carbon backbone supply and ‘‘normal’’ nitrate assimilation while certain amino acids are impaired in synthesis (cysteine) asks for an integration of carbon and nitrogen metabolism with sulphate metabolism (Hesse et al., 2004a; Kopriva and Rennenberg, 2004). As well, genes as metabolites of the major nitrogen-transporting amino acids, glutamine and asparagine, are usually increased under severe S starvation conditions to compensate the shift in balance of N/S towards N and to prevent ammonia intoxication of the plant (Nikiforova et al, 2005b; 2006). Here, however, this was true only for glutamine.

Amino acid levels from this study together with expression pattern of accompanied genes were used by Nikiforova et al. (2006) for reviewing the effect of sulphur availability on the integrity of amino acid biosynthesis in plants.

Metabolite profiling revealed new metabolites, which could be involved in plant responses to sulphur deficiency stress. Interestingly, two metabolites from vitamin E biosynthesis pathway were changed under S limited conditions. γ-tocopherol, a direct precursor of α-tocopherol (the major vitamin E compound found in leaf chloroplasts; Munne-Bosh, 2005), and its precursor, 2,3-dimethyl-5-phytylquinol, were both elevated under –S conditions (in the 2nd experiment), when compared to full nutrition conditions (Appendix A). Tocopherols are presumed to be important antioxidants deactivating photosynthesis-derived reactive oxygen species and preventing the propagation of lipid peroxidation by scavenging lipid peroxyl radicals in thylakoid membranes. It is generally assumed that increases of α-tocopherol contribute to plant stress tolerance, while decreased levels favor oxidative damage. Recent studies indicate that tocopherols together with other antioxidants (such as: ascorbate, glutathione, carotenoids, isoprenoids, flavonoids and enzymatic antioxidants) afford to ensure the adequate protection to the photosynthetic apparatus and help plants to withstand environmental stress (Munne-Bosh, 2005; Hollaender-Czytko et al, 2005).

Observation of changes in metabolite levels after sulphate re-supply leads to an interesting conclusion. Metabolites from the primary assimilation pathway, such as cysteine and serine are responding rapidly to the changes of sulphate level in the environment – already after 30 minutes of SO_4^{2-} re-addition their pools start to be restored to that from control conditions (Fig 3-1 and 3-4). The

same observation concerns γ-Glu-Cys and glutathione (Fig 3-1). However, none of metabolites located more downstream in the pathway, such as cystathionine, homocysteine, methionine and also putrescine change their levels after 30 minutes of re-supplementation, some appeared to be slightly restored in content after 3 hours (Fig 3-1 and 3-4).

In conclusion, the necessity to maintain viability in conditions where sulphur, a crucially important macronutrient, is deficient results in a systemic internal rebalancing of plant metabolism. This is reflected by decreased or increased levels of distinct metabolites in sulphur-deficient plants. The integrity of the amino acid biosynthesis system is kept, though shifted. The shift in balance and regulation of amino acid biosynthesis allows the plant to readjust its homeostasis and to remain viable and produce seeds for dispersal.

4.A.4 S-regulated TF candidate genes were identified by using several strictly defined selection criteria

Comparison of quantitative RT-PCR data from all nutrient approaches revealed a different amount of TF genes which expression was found to be affected. Nitrate deprivation strongly (10-fold) affected 45 TF genes (Czechowski, personal communication), while phosphate starvation resulted in strong (10-fold) changes of expression of 8 TF genes. 19 TF genes exceeded the induction threshold by the level of 10 upon sulphate limited conditions. Thus, we finally decided to analyse TF genes, which were changed in their expression more than 5 or less than 0,2-fold, compared to respective control conditions. Approximately 7,9% (112 TF genes out of 1417) of the *Arabidopsis* TFs were significantly (more than 5-fold) affected in all tested conditions (Appendix C), while 5,4% (76 TF genes out of 1417) were changed in expression over threshold 5 in sulphur depleted conditions.

The distribution between TF which were positively and negatively regulated was strongly shifted towards the first group. Sixty three TF genes were found to be induced more than 5-fold after S deprivation, while only thirteen genes were found to be repressed more than 5-fold after 48 h of S-deprivation.

Re-supplementation of cultures with sulphate allowed to discriminate between changes specific and unspecific to sulphate and, in consequence, to identify TF genes that may specifically induce or repress changes in expression of genes that allow plants to adapt to changes in sulphate availability. Of 76 TF genes, which had been strongly affected by S deprivation, only 12 responded to sulphate re-addition at least in the time frame (30 minutes to 3 hours) studied here. After 30 min or/and 3h of S re-supply, nine genes induced by S depravation responded in a negative manner (more than 5-fold repression) and 3 genes repressed by S deprivation responded in a positive (more than 5-fold induction) way. Most of the TF genes that were induced or repressed by S-deprivation did not respond significantly to subsequent SO_4^{2-} re-supply, however, only around 10 TF genes clearly did not change their expression after sulphate re-addition. Such genes were considered either to act more downstream in the miltifactiorial regulation network or be a part of a pleiotropic response. Other TF, although did not reach the same expression level as in full nutrition, exhibited a slow tendency for being re-induced or re-repressed, which, nevertheless, was considered as a proof for their specificity

in response to sulphate deprivation. Among all possible expression patterns exhibited by TF genes in all four experimental conditions, those with rapid and contrary changes in expression under S-limited conditions and further re-supplementation was considered to be favourable in terms of sulphate specificity and were thus chosen for further analysis.

There was a satisfactory reproducibility for the sulphate responding TFs in a biological replica (Experiment 2). Although only 14 of the 58 genes responded to S-deprivation in the same way and at similar magnitude (>5 fold change in the transcript level), 21 other genes showed the same tendency of response but at lower level (2-5 fold) (Table 3-7). Eight genes responded to S-starvation in the opposite way to that observed in the first experiment. Such variability seems to be reasonable, when taking into account, that the 2nd experiment was less severe in terms of sulphur starvation. This could explain why some genes responded similarly, but to the less extend. Possible explanation for the high variation in TF gene expression after S-deprivation is that many S-responding TF genes were undetected in one or the other condition (expression ratio based on C_T value of 40) in the 1st experiment. qRT-PCR can fail not only due to lack of transcript under given condition but also because of technical problems (e.g. mispipetting of the reagents, problems with reading fluorescence from a given well, inhibitors of *Taq* polymerase, lowering given PCR reaction efficiency, etc.). In fact, four S-responding genes, which failed to be reproduced in the second experiment, have expression ratios based on C_T value 40.

There was a good agreement with the data obtained form Affymetrix arrays and qRT-PCR. Of 58 preliminary chosen S-responsive TF genes, 41 exhibited similar expression patterns reported by both techniques (data not shown). Only five TF were not confirmed, seven exhibited contradictory results and another five were not arrayed on the ATH1 chip. Finally, 20 TF genes possibly S-specific and confirmed by Affymetrix data were selected as strong candidates. It is apparent from Table 3-7, which shows 20 S-regulated TF candidate genes, that data obtained from Affymetrix arrays are generally consistent with that obtained by qRT-PCR. However, fifteen out of 20 candidate genes identified by qRT-PCR were considered on ATH1 array as “absent” (by MAS5 software in at least one experimental condition). Another three were not arrayed on ATH1 chip. This emphasises again the superiority of qPCR as a technique to detect rare transcripts, that cannot be measured by hybridisation based techniques.

4.A.5 An overview of selected S-regulated transcription factors

A broader look at S-regulated genes showed a clear and significant overrepresentation of the MYB-family TF genes with 18 members up-regulated more than 5 times (Fig. 3-9). A reason for that might be the fact that the MYB superfamily has the largest number of members of any *Arabidopsis* gene family with 198 genes identified (Riechmann and Ratcliffe, 2000), but also it could be explained by a wide range of processes MYB TFs are involved in. Notably, all 18 S-responsive members belong to the R2R3-MYB subfamily. Indeed, they were reported to be involved in many physiological and biochemical processes, which could be required to cope with S-deprivation stress, such as the regulation of secondary metabolism (Paz-Ares et al., 1987; Bender and Fink, 1998; Hoeren et al., 1998; Borevitz et al., 2000; Jin et al., 2000; Nesi et al., 2001; Baudry et al., 2004), including PAP1 and

PAP2 TFs involved in transcriptional activation of production of anthocyanin pigment. Some R2R3-MYB TFs were also involved in various abiotic stress responses, such as dehydratation responses (Urao et al., 1993), response to cold (Stockinger et al., 2001), salt stress (Nagaoka and Takano, 2003), response to wounding and osmotic stress (Denekamp and Smeekens, 2003). Moreover, a conserved MYB TF was found to be involved in phosphate starvation signaling both in algae and vascular plants (Rubio et al., 2001), therefore involvement of these TF in sulphate starvation stress is conceivable.

A number of S-regulated TFs identified in this study belong to TF families that have been implicated in developmental regulation, such as MADS-box TF genes (9 genes induced), NAC (6 genes induced) and AP2/EREBP TFs (6 genes induced) (Fig. 3-9). These TF families are associated mainly with flower, leaf and roots development (Alvarez-Buylla et al., 2000a; Alvarez-Buylla et al., 2000b; Olsen et al., 2005). Arabidopsis *AP2*, the most well-studied gene in AP2/EREBP family, encodes a putative plays a central role in the establishment of the floral meristem (Irish and Sussex 1990; Huala and Sussex 1992; Bowman et al. 1993; Schultz and Haughn 1993; Shannon and Meekswagner 1993), the specification of floral organ identity (Komaki et al. 1988; Bowman, Smyth, and Meyerowitz 1989; Kunst et al. 1989), and the regulation of floral homeotic gene expression (Bowman, Drews, and Meyerowitz 1991; Drews, Bowman, and Meyerowitz 1991; Jack, Brockman, and Meyerowitz 1992; Mandel et al. 1992) in *Arabidopsis*. The *ANT* gene regulates ovule development and floral organ growth (Elliott et al. 1996; Klucher et al. 1996). Genetic evidence shows that the MADS-box gene *ANR1* (*At2g14210*) is required to mediate changes in root architecture in response to NO_3^- availability. *ANR1* expression was induced in roots within 30 min of adding NO_3^- to *Arabidopsis* seedlings (Zhang and Forde, 1998). It is interesting to speculate that some of these TFs may integrate S-signals into plant development programmes.

WRKY transcription factors have so far been associated mainly with plant defence responses such as: defense against attack from pathogenic bacteria (Du and Chen, 2000; Deslandes et al., 2002; Chen et al., 2002; Chen and Chen, 2002; Dong et al., 2003), fungi (Chen et al., 2002), viruses (Wang et al., 1998; Yang et al., 1999; Chen et al., 2002), and oomycetes (Chen et al., 2002; Kalde et al., 2003). Further, WRKY genes are implicated in responses to the abiotic stresses of wounding, being induced by signaling substances such as salicylic acid (Hara et al., 2000; Cheong et al., 2002), the combination of drought and heat (Rizhsky et al., 2002), and cold (Huang and Duman, 2002). Six TF genes induced by S-deprivation (Fig. 3-9) may suggest that WRKY TFs fulfil biological functions beyond responding to biotic stress and are involved in nutritional signaling.

The set of S-regulated TF genes contained members of TF families that have been implicated in hormone response pathways, including: AP2/EREBP (ABA and ethylene responsive), MYB (ABA responsive), bZIP (ABA and gibberellins), NAC (auxin responsive) (Riechmann, 2002). This results may indicate possible cross talk between hormone and sulphate-signalling pathways.

Although a bHLH family is a large TF family in plants, only four bHLH genes were found to be induced upon S-starvation, another four were down-regulated (Fig. 3-9). Interestingly, the Cbf1 protein, which is involved in transcriptional activation of sulphur metabolism genes in yeast, contains a basic helix-loop-helix (bHLH) domain (Cai and Davies, 1990; Mellor et al., 1990; 1991). Cbf1 binds to

the *cis*-acting element of the yeast homocysteine synthase gene (*MET25*) in a heteromeric transcription activation complex together with Met4 and Met28, which both posses bZIP domains (Kuras et al., 1996; 1997). However, among all S-responsive TF genes found in this approach, only two genes belong to the bZIP TF family. No orthologus genes for *Cbf1*, *Met4* and *Met28* have been found in higher plants, however, involvement of bHLH and bZIP TFs in S-signaling plants is still possible.

4.A.6 Responses to other macronutrients allowed the identification of TF candidates which responded specifically to S-deprivation

Sterile liquid cultures and constant light were used to minimize the diurnal changes and other unspecific influences of environmental factors. Such artificial system of plant cultivation allowed the researcher to strictly manipulate the conditions and, thus, to better control them. Several nutritional and other stress-conditions experiments were performed using the same axenic culture system in which plants were cultivated in similar conditions but exposed to changes in other macronutrients, and to abiotic stresses, including salt and osmotic stress. This provided a large and comparable set of data, which all contributors could profit from.

An additional way to ensure that changes in expression of candidate TF genes were specific to changes in S-nutrition, was comparing TF transcript data between series of other experiments (Table 3-8). This set of data was kindly provided by collaborators from Molecular Plant Nutrition and Molecular Genomics Group (as mentioned in Results section).

Most of the 20 S-regulated TF responded also to other than S-deprivation stimuli, however, a magnitude of response was differentially pronounced for different stresses (Table 3-8). This confirms involvement of TFs in a multifactorial response network (Broun, 2004), in this case, probably a general stress response network, in which particular TFs play a role more or less attributed to a certain stress stimuli. Eleven TF responded significantly (more than 5-fold) to changes in either P and N nutrition (Table 3-8) and thus are considered to be a part of nutritional stress response network. There was a bigger overlap between TF genes induced by nitrate and sulphate than sulphate and phosphate. This could result simply from the fact that lack of nitrogen, which is more abundant in plant tissues than S and P, is a stronger stress stimuli than limitations of sulphur or phosphorus. Another explanation would assume that sulphate- and nitrate-responsible factors are closely related in the multifactorial response network and, thus, co-regulated. Existence of such a network of common nutrient-responses confirms also fact that TF genes positively and negatively induced by S-deprivation exhibited the same tendency with regard to N- and P-limitations (Table 3-8), with a very few exceptions only.

These comparisons resulted in the identification of a few genes that responded to general abiotic stresses, rather than specifically to sulphate, i.e. *PAP1* (*At1g56650*), *PAP2* (*At1g66390;* see Appendix C) *AtMYB52*, *AtMYB53*, *AtMYB105*, *AtMYB9*, and others. *PAP1* and *PAP2* responded to deprivation of each of macronutrients tested, and also to salt stress. Genetic studies suggest that PAP1 and PAP2 are involved in the transcriptional activation of anthocyanin and flavonoid biosynthesis. Activation tagged *Arabidopsis* mutants overexpressing either of these genes exhibited

purple pigmentation in all organs (Borevitz et al., 2000). It is well known that regulators of anthocyanin biosynthesis respond generally to stress conditions that compromise growth of plants. It is worth mentioning, that although upon S-limited conditions applied here, *Arabidopsis* seedlings exhibited no anthocyanin accumulation, both these genes were strongly induced in –S. This could be explained by the fact that transcriptional activation of certain target genes and processes begins prior to the molecular or physiological effect (in this case – purple pigmentation) which is the outcome of these processes. We do not know, whether a prolonged S-starvation would result in anthocyanin accumulation in starved seedlings. One could assume so, as there was a high demand for PAP1 and PAP2 proteins, which manifested in strong overexpression of respective genes.

Finally, we selected a set of 8 genes that represents possibly specific and strong responses to sulphate deprivation: *AtWRKY43*, *At2g38340*, *AtWRKY24*, *At1g72570*, *AtbHLH052*, *AtbHLH028*/*ERF2*, *ATAF1* and *At4g33960*.

Summarising, the 'rapid and contrary' expression pattern under S-limited conditions and further re-supplementation, the specificity in reaction to changes of sulphate level (compared to other nutrients and abiotic stresses), satisfactory reproducibility of expression pattern in independently replicated experiment, and finally agreement with respective data obtained from Affymetrix arrays were considered in the process of selection of the final 8 S-responsive TF candidate genes.

4.A.7 A reverse genetic approach with using T-DNA insertion mutant lines was taken for preliminary functional characterization of TFs

A loss of function approach was taken for functional characterisation of TF genes selected in the short term S-starvation experiment. At least two different homozygous knock out lines were obtained for six candidate TF genes. Lack of the transcript or at least strong reduction of transcript levels was detected for three out of six selected mutant lines (Table 3-9).

None of the homozygous KO lines showed visible aberrant phenotypes when grown on soil, or when screened for germination ratio, flowering time and changes of root architecture. One possible reason for that might be connected to the method which was chosen for functional characterisation of selected candidates. Even if the homozygous KO lines lack of functional TF transcript, many may exhibit no phenotype aberration because of high functional redundancy among *Arabidopsis* TF genes. A cross-comparison of TFs, in *Arabidopsis* revealed that, closely related genes can be found in nearly half of the major TF families (Riechmann et al., 2000). Pairs or groups of closely related genes correspond to duplication on different chromosomes (~65% of cases), duplications on the same chromosome but at very large distances (~22%) and less frequently to tandem repeats (~13%) (Riechmann et al., 2000; Riechmann, 2002). Clusters of three or more homologous TFs are very rare in the genome (Riechmann et al., 2000; Riechmann, 2002). Thus, insertional mutagenesis into one TF gene will generally not affect the expression of its most closely related homologue, which may substitute for its loss. The extent of functional redundancy among TFs is illustrated by several studies on MADS-box genes. Sequence analysis of *Arabidopsis* MADS-box genes suggests that more than 40% may have (partially) redundant functions (Davies et al., 1999; Liljegren et al., 2000; Pelaz et al.,

2000). Conserved expression of gene pairs might represent an evolutionally favoured backup system that avoids loss of vital functions due to spontaneous mutations.

In fact, such possibility for the functional substitution was revealed to exist for the *WRKY24* gene (*At5g41570*). A duplicated twin of this gene was found on the first chromosome, in *At1g64090* loci. Duplicated chromosome segments were identified using the final TIGR Arabidopsis genome annotation release version 5.0, where the *At1g64090* is annotated as a *WRKY* gene. In this case occurrence of functional substitution seems to be possible. However, according to other databases (http://arabidopsis.med.ohio-state.edu/AtTFDB/ or http://www.arabidopsis.org/), the *At1g64090* gene product is annotated as a reticulon family protein of unknown function. It is possible that during evolution processes one of these genes gained new function. Divergence of formerly identical regulatory elements by mutation and subsequent natural selection presumably leads to new biological roles for genes/proteins derived from a single progenitor by duplication, and may be a route to increase fitness and adaptation to a given environment.

Such functional substitution by a closely related homologue probably did not occur in case of two other S-induced TF genes, *At1g34670* and *At4g33960*, as they were not found to be duplicated within the *Arabidopsis* genome.

It is also possible that investigated gene knockouts do not have any impact on the morphological phenotype and that simply no nutritionally specific phenotype alteration exists. First, all typical symptoms of sulphur deprivation in plants, such as: stunted growth, chlorosis, accumulation of anthocyanins in the leaves, increase in root biomass and pronounced lateral root growth (Nikiforova et al., 2003), are typical for many biotic and abiotic stresses. Secondly, would it be reasonable to expect any phenotype changes from mutants grown in normal conditions (soil, full nutrition medium), as it is known that a mutant phenotype may not be discovered unless a particular stress is present during the analysis (Riechmann and Meyerowitz, 1998)? Besides that, although estimates suggest that the *Arabidopsis* genome has been saturated with T-DNA and transposable element insertions, relatively few informative knockouts that provide a clue to gene function have been reported (Bouche and Bouchez, 2001). Several examples for gene knockouts in plants have been reported in which a defect in a certain transcription factor gene failed to generate informative phenotypes at the macroscopic level (Baima et al., 2001; Pontier et al., 2001; van der Graaff et al., 2002; Fan and Dong, 2002). Only when multiple transcription factors were knocked out, could informative phenotypes be obtained (Liljegren et al., 2000; Eshed et al., 2001; Kumaran et al., 2002). Also large-scale knockout analyses of *Saccharomyces cerevisiae* have been recently carried out using insertion mutagenesis and provided similar conclusion (Giaever et al., 2002). Another study on *Caenorhabditis elegans* reported knockouts for 86% of all predicted genes but only ten percent of these knockouts showed phenotypical differences (Kamath et al., 2003). One of the key observations made during these experiments was that only a low percentage of mutants showed a phenotype. As a consequence, informative knockouts providing a clue to gene function are limited (Bouche and Bouchez, 2001). Finally, it is possible that the lack of phenotypes in knockouts could be explained by our inability to detect small phenotypic changes (Zhang, 2003).

Another potential problem might be the specific participation of transcription factors in regulatory circuits that control responses to sulphur stress. Again, a mutant phenotype may not be discovered unless this particular stress is present during the analysis (Riechmann and Meyerowitz, 1998). Thus, to demonstrate involvement of selected transcription factors in sulphur assimilation, axenically grown plants were subjected to sulphur starvation. Although levels of internal sulphate decreased in all lines tested from 5 to 8% of the full nutrition levels, neither mutant nor wild type lines showed any known visible indications of sulphur starvation compared to respective control plants, which had not been exposed to this stress. However, it is still possible, that longer cultivation under S-limited conditions could give a chance for phonotypical alterations occurrence in KO lines, especially, when growing them over their whole life-time i.e. hydroponically. However, it was considered to be more promising to search for alterations in their physiological phenotype.

It is also to early to conclude that mutations in TFs of interest are responsible for observed physiological phenotype changes, because mutants have not been backcrossed to the wild type to remove any additional T-DNA insertions in other than investigated loci. Multiple insertions are common in the T-DNA populations used here (Alonso et al., 2003). To overcome this problem, we decided to study, if possible, at least two different SALK lines for preliminary functional characterisation of one and the same TF gene. This, however, was possible only for the *WRKY24* gene, for which we used SALK_119740 and SALK_008183. For other TF genes we used at least two different homozygous lines of one and the same SALK line. This also gave us a possibility to confront results obtained for different homozygous lines possessing T-DNA insertion in the same TF gene. We assumed that, if additional T-DNA insertion in other than investigated loci occurred, it will influence the morphological or/and physiological phenotype independently. Thus, as most reliable results we considered only those, which were obtained similar for both homozygous lines with the same TF gene impaired, as it was the case for lines 5-1-2 and 5-1-7. Differences in physiological changes in lines SALK_119740 and SALK_008183 (two SALK lines for *WRKY24*) line might be caused by independent T-DNA insertion, presumably occurred in the latter one.

The content of internal anions was determined to check, whether the impaired genes influenced the nutrient status of SALK mutant plants. The sulphate level was rather constant within lines and comparable to the wild type level (Fig. 3-16). However, both homozygous SALK_119740 lines (5-1-2 and 5-1-7) revealed decreases significantly stronger than Col-0 line in the internal SO_4^{2-} content, when analysing S-starved plants. Also, in normal conditions, these two lines exhibited slightly lower sulphate levels, when compared to wild type. Sulphate amount significantly higher than in starved wild type plants was detected in the line 9-1-9; also in control conditions this line revealed slightly elevated sulphate content. Unfortunately, the line 9-1-1 did not exhibited similar changes with regard to the internal sulphate levels.

Decreased contents of all four thiols were revealed for both homozygous SALK_119740 lines (5-1-2 and 5-1-7), in both normal and –S conditions (Fig. 3-15). Second KO line analysed for the *WRKY24* gene, the SALK_008183 line, did not provide a consistent picture with regard to the thiol level. This misleading result could be, however, correlated with not complete loss of function of the target gene detected for the 5-4-2 line (see table 3-9). In contrast to that, both homozygous

SALK_131752 lines (9-1-1 and 9-1-9) presented elevated amounts of thiols, when compared to wild type, in both normal and –S conditions (Fig. 3-15). The highest increase was detected for homocysteine, which was 1,7 times higher in both lines grown on full nutrition medium, compared to Col-0, and almost 4 and 3-fold higher than in wild type under –S in the lines 9-1-1 and 9-1-9, respectively.

Target expression analysis on SALK T-DNA insertion lines using quantitative RT-PCR revealed increased transcripts levels of many genes from sulphate uptake and assimilation (Table 3-10). Among the high affinity sulphate transporters, the *SULTR 1;2* was up-regulated more than 2-fold in 5-4-2, 5-4-18, 9-1-1 and 9-1-9 lines, while *SULTR 1;1* expression remained unaffected in all of the SALK mutants lines. It is worth mentioning that the only gene, which was found to be down-regulated in all transgenic lines tested, was the *SULTR 1;3*. Among genes acting downstream in the pathway, the group of OAS (thiol)lyase genes was revealed to be over-expressed in most of the tested lines. Interestingly, genes belonging to the APS reductase family were down-regulated exclusively in lines 17-1-3 and 17-1-8, while in others were mainly unchanged.

Expression pattern of genes from sulphate uptake and assimilation pathway suggests that selected S-induced TF genes act as their repressors. Especially *WRKY24* and *MYB93*, when impaired, seem to stimulate induction of many genes from S-metabolism. Why then *WRKY24* and *MYB93* were overproduced when wild type plants were subjected to the sulphur limitation? Two explanations of this contradictory phenomenon appear to be reasonable. First, WRKY24 and MYB93 could act as the positive regulators of *SULTR1;3*, as it is the only gene down-regulated in these mutants lines. As *SULTR1;3* belongs to the high affinity sulphate transporter genes family, one can assume, that even slight decrease in its transcript abundance can have a significant impact on the sulphate uptake. To compensate this, *SULTR1;2* appeared to be induced in expression in KO lines of *WRKY24* and *MYB93*. Moreover, expression levels of *SULTR1;3* and *SULTR1;2* seem to be mutually regulated in these lines. Elevated transcript abundancies of all other genes acting downstream in the S-assimilation pathway would result from the diminished sulphate uptake. Although mostly wild type-like internal sulphate levels in *WRKY24* and *MYB93* KO lines does not support this idea, decreased content of cysteine in lines 5-1-2 and 5-1-7 suggest, that such explanation might be possible. Why lines 9-1-1 and 9-1-9 revealed opposite tendency with regard to cysteine content, remains unknown.

Another explanation of the above mentioned result assumes that TFs investigated in short term S-starvation approach are involved in a complex multifactorial regulatory network, which, in fact, is very likely to occur for transcription factors (Broun, 2004). In this regulatory network, *WRKY24* and *MYB93* would act as superior factors regulating other transcription factors directly involved in the regulation of S-metabolism genes. These direct regulators would act as inducers, while secondary regulators, *WRKY24* and *MYB93*, would have a function as repressor (Fig. 4-2). Thus, in knock out lines, disruption of the *WRKY24* and *MYB93* gene function caused induction of primary factors, which in turn induced target genes from sulphate uptake and assimilation. In wild type plants, a balance between repressors and inducers would maintain the expression of genes from sulphate metabolism at the steady-state level and allow to adjust it to the changes in internal sulphate concentration.

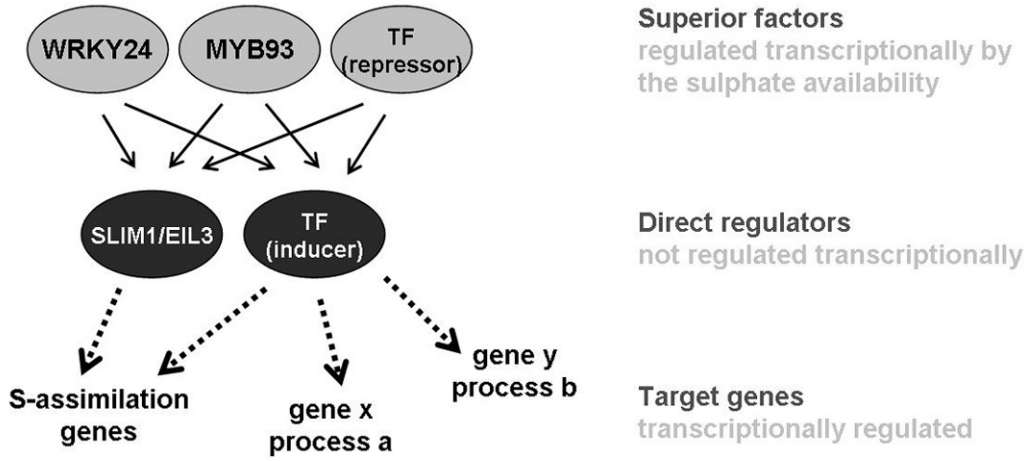


Fig 4-2 Speculative model describing the regulation of expression of S-assimilation genes by S-induced transcription factors. WRKY24 and MYB93 act as superior factors regulating other transcription factors directly involved in the regulation of S-metabolism genes. The direct regulators act as inducers, while secondary regulators, WRKY24 and MYB93, have a function as repressor. Solid arrows indicate protein-protein interactions, dashed arrows indicate protein-DNA interactions involved in transcriptional regulation of target genes.

Recently, the SLIM1/EIL3 transcription factor has been identified to co-regulate sulphate uptake and sulphur recycling processes in *Arabidopsis* (Maruyama-Nakashita et al. 2006). Especially, the expression of genes, which are known to be used by S-deprived *Arabidopsis* plants to maximize their sulphur use efficiencies in root tissues, such as: SULTR1;1, SULTR1;2, SULTR4;1 and SULTR4;2, was shown to be SLIM1-dependent. These pathways were almost completely turned down in *slim1* mutants, suggesting that SLIM1 may act as their positive regulator (Fig. 4-2). Although the SLIM1 was the first reported TF regulating the assimilatory sulphur metabolism in higher plants (Maruyama-Nakashita et al. 2006), in proposed model of regulation, it would fulfill only the direct regulator functions. Thus, WRKY24 and MYB93 were first identified superior factors which presumably are part of the same S-assimilation regulatory system. In this case, revealing their mutual interdependences by the response network reconstruction analysis would follow expectations (see chapter 2.5.4). However, neither WRKY24 nor MYB93 did not pass imposed selection criteria and were not present in the final dataset used for the biosystem response network reconstruction.

As mentioned before, SLIM1 was shown not to be regulated transcriptionally under S-limited conditions, either by Maruyama-Nakashita et al. (2006) or in this study (data not shown). This would suggest, that, according to the proposed model of regulation, interactions between repressors and inducers does not occur at the expressional level. Moreover, the tissue specific localisation of SLIM1 was monitored using a GFP-SLIM1 fusion protein, which levels were shown to be unaffected by the sulphur availability (Maruyama-Nakashita et al. 2006). Thus, as both, the mRNA and the protein abundance were not modulated under –S, regulation of the SLIM1 activity by protein-protein interactions between SLIM1 and its probable repressors is very likely to occur. On the other hand, the superior factors, WRKY24 and MYB23, identified in this study, are regulated strongly at the transcriptional level by changes in sulphate availability. Hence, according to the proposed model of regulation, we can generalize that regulators positioned more upstream in the hierarchy of interactions are commonly regulated at their expressional level by changes of sulphate conditions, while direct regulators are transcriptionally insensitive to the changes of sulphate availability. This might be

explained by their indispensability in the regulation mechanism in which they are involved. Indeed, the SLIM1/EIL3, but no other EIL proteins from *Arabidopsis*, was shown to regulate uniquely sulphate uptake and acquisition, as only this protein was able to restore the sulphur limitation response-less phenotype of *slim1* mutants (Maruyama-Nakashita et al. 2006). The abundance of WRKY24, MYB93 and other superior factors would be transcriptionally regulated by other upstream factors or directly by mechanisms involved in the sulphate level-sensing signal cascades, which are yet to be understood.

Similar changes in transcript levels of many genes from sulphate uptake and assimilation pathway which were observed in *WRKY24* and *MYB93* knock out lines suggest that both, WRKY24 and MYB93, might have a similar influence on these genes and, thus, a redundant function. However, differences in metabolite levels observed between *WRKY24* and *MYB93* knock out lines does not support this idea. Especially, levels of thiols, which are generally elevated in *WRKY24* knock out lines and decreased in *MYB93* mutants, indicate involvement of other regulatory mechanisms existing in plants. These mechanisms, involving unknown factors or interactions, would act independently from WRKY24 and MYB93 TF and, therefore, might be made responsible for differences observed between knock out lines. Existence of such multiple regulatory mechanisms of S-assimilation genes was postulated also by Maruyama-Nakashita et al. (2006). Although transcripts of several sulfate transporters were modulated by SLIM1, the effects of this transcription factor was not exclusive for the control of SULTR1;1 that also follows –S-responsive regulation under a previously identified *cis*-acting element, SURE (Maruyama-Nakashita et al., 2005). In fact, SULTR1;1 mRNA was partially induced by –S even in the *slim1* mutants (Maruyama-Nakashita et al. 2006). By contrast, absence of SURE from the promoter region of SULTR1;2 suggests that this major sulfate uptake facilitator is controlled predominantly by SLIM1 under –S conditions. Corollary of the SLIM1-mediated regulation of SULTR1;2, its sulfate uptake activity was significantly decreased by *slim1* mutations. As –S-responsive gene expression is critical for plant survival in the –S condition, it is reasonable that plants have evolved multiplex regulatory mechanisms to control gene expression. Further identification of signaling components should unravel the full set of mechanisms underlying this hypothesis.

As mentioned in the chapter 3.A.5.7, according to a recent knowledge, the molecular function of the *At4g33960* gene remains unknown, giving no evidence for its involvement in the regulation of transcriptional processes. Although the *At4g33960* gene did not revealed a strong influence on the expression of genes from sulphate uptake and assimilation pathway, when impaired, *APS reductase* family genes and *SULTR1;3* were down-regulated in *At4g33960* knock out lines. This would implement possible regulatory function of the *At4g33960* gene product, which could act as a direct inducer of the SULTR1;3 gene and genes of APS reductases. This challenging result may suggest, that a weak homology of the expressed protein encoded by the *At4g33960* gene to a MADS-box region allows this protein to work as an transcription factor.

4.B Functional characterisation of two APETALA 2 TF genes: *TOE1* and *TOE2*

4.B.1 AP2 transcription factors TOE1 and TOE2 promote conversion from cysteine to methionine

TOE1 (*At2g28550*) and *TOE2* (*At5g60120*) AP2 genes were selected as candidate transcriptional regulators in a long term sulphur-depletion experiment, in which the expression behaviour of approximately 7200 *Arabidopsis* genes was examined using macroarray hybridization method (Nikiforova et al., 2003).

For functional characterisation of selected S-regulated TF genes, a gain-of-function approach was taken, in which the *TOE1* and *TOE2* genes were overexpressed under a strong constitutive promoter, the cauliflower mosaic virus 35S promoter.

T2 lines containing the *35S-At2g28550* construct (# 63) and lines containing the *35S-At5g60120* construct (# 70) were checked for overexpression. qRT-PCR reactions revealed from 3 to 13-fold overexpression of both genes in roots and leaves of respective lines (Fig. 3-17).

Both *35S-TOE1* and *35S-TOE2* lines showed visible phenotype alterations when grown on soil, vertical plates, and in hydroponics. The majority of plants containing the *35S-At2g28550* construct (# 63) were strongly delayed in flowering and showed large rosette leaves, while most of the plants containing the *35S-At5g60120* construct (# 70) were flowering earlier than wild type and had smaller rosette leaves (Fig. 3-18 – Fig. 3-20, Fig. 3-22 and Fig. 3-29). In fact, phenotypical alterations for the *35S-TOE1* line observed in this study match observations reported by Aukerman and Sakai (2003), who determined that overexpression of *TOE1* causes late flowering. It was also reported in that study that homozygous T-DNA insertion mutants of *TOE1* (*toe1-2*) were slightly early flowering relatively to the wild type, whereas mutants of *TOE2* (*toe2-1*) were not significantly early flowering. However, the *toe1-2 toe2-1* double mutant was earlier flowering than either individual mutant, suggesting that the genes have overlapping function. Based on this results authors proposed that at least some of the *AP2*-like genes, including *TOE1* and *TOE2*, function as floral repressors. However, it has not been proven that overexpressing of *TOE2* causes the early flowering phenotype. Moreover, results provided by the study of *toe2-1* suggest that it might not be the case, which would match phenotype observations for the *35S-TOE2* line done in this work. None of observed phenotype alterations, however, provides any direct indication for connection with sulphur metabolism. Thus, an investigation of the physiological phenotype was of high interest.

Following the idea, that some phenotypical alterations, especially those connected to nutritional metabolism, might not appear unless particular stress conditions are applied (Riechmann and Meyerowitz, 1998), we subjected hydroponically cultivated *35S-TOE1* and *35S-TOE2* lines to sulphur starvation. After ten days in S-limited conditions, some *35S-TOE1* lines exhibited an over 40-fold accumulation of antocyanins and, especially the line 63-10, retardation in growth, when compared to starved Col-0 plants (Fig. 3-29 and Fig. 3-30).

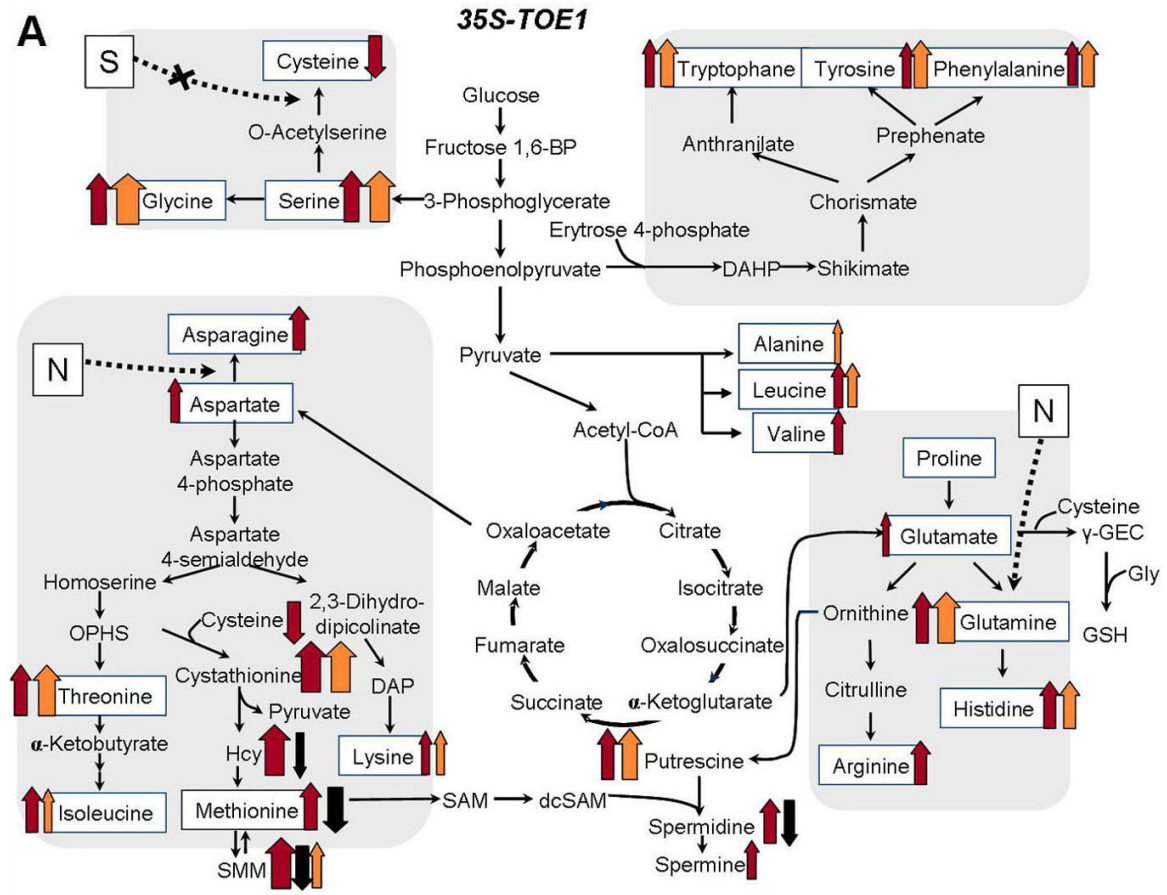
The sulphate level was rather constant within all plant lines. Only few lines exhibited significantly lower sulphate amounts, when grown in normal conditions (Fig. 3-23). Lines 63-5 and 63-12 revealed significantly lowered sulphate levels in both leaves and roots, when compared to wild type, while the line 70-30 had less sulphate in roots. An interesting observation was made for 35S-AP2 plants, when grown in S-limited conditions: all 35S-AP2 lines exhibited significantly lower sulphate levels in leaves. This could be explained in two ways: either the sulphate uptake was reduced, due to unknown regulation mechanism acting/enhanced in these transgenic plants, or it was a higher demand for sulphate and the consumption rate exceeded the rate of its uptake. Consequently, the 'sulphur-deficiency-sensing' in S-starved 35S-AP2 plants was stronger than it was in wild type plants starved for sulphate.

Despite that thiol levels did not show a very consistent picture, for some lines common changes in thiols were observed. Such lines were selected for further analysis. Biggest changes were found for homocysteine levels, which were strongly and significantly higher in lines 63-10 and 63-12, compared to Col-0, in both leaves and roots (Fig. 3-24). However, when growing plants in S-limited conditions, the content of homocysteine decreased even stronger, than it was observed in starved wild type plants. Similar, but slightly milder changes were found for homocysteine contents in the line 70-30. Cysteine levels were found to be lower in leaves of lines 63-10, 63-12 and 70-30, compared to Col-0, when grown in normal conditions (Fig. 3-24). In sulphur limited conditions, cysteine decreased stronger in roots of *35S-TOE1* and *35S-TOE2* plants.

Decreased levels of cysteine in leaves of 35S-AP2 plants can be explained by strong down-regulation of expression of most genes from sulphate uptake and assimilation pathway, which was found to happen almost exclusively in this tissue (Table 3-12). In contrast to that, these down-regulated in leaves genes were found to be highly overexpressed in roots, what would indicated a high demand for sulphate and cysteine sensed by those plants. Interestingly, among *SULTR* genes, the highest induction exhibited *SULTR3;2*, expressed exclusively in roots, and *SULTR2;1*, the highly S-regulated transporter expressed in vascular tissues.

Strong differences in most amino acid contents were found between wild type plants and all four 35S-AP2 lines (Fig. 3-25 – Fig. 3-28). In general, most of amino acids were increased in content in *35S-TOE1* lines (especially in 63-10 and 63-12), often in both leaves and roots, while in the *35S-TOE2* line (70-30) these differences were similar but milder. These changes were accompanied by increased levels of polyamines, especially putrescine and spermidine, which were often significantly higher in leaves and roots of 35S-AP2 plants, compared to Col-0, when grown in S-sufficient conditions (Fig. 3-31). Superposition of changes in metabolite pools on the amino acid biosynthetic pathways for *35S-TOE1* and *35S-TOE2* plants are depicted in figure 4-3, A and B, respectively.

Although sulphate levels in 35S-AP2 plants were mostly unaffected in normal conditions, observation on metabolite levels indicates, that changes in metabolites in these lines are very much mimicking the changes, which were observed in wild type plants starved for sulphate (see Fig. 4-1 and Nikiforova et al., 2003; 2006). This includes decrease in cysteine content, accumulation of serine and


A
35S-TOE1
S
Cysteine
O-Acetylserine
Glycine
Serine
Glucose
Fructose 1,6-BP
3-Phosphoglycerate
Phosphoenolpyruvate
Erytrose 4-phosphate
DAHP
Shikimate
Chorismate
Anthranilate
Prephenate
Tryptophane
Tyrosine
Phenylalanine
N
Asparagine
Aspartate
Aspartate 4-phosphate
Aspartate 4-semialdehyde
Homoserine
OPHS
Cysteine
2,3-Dihydro-dipicolinate
Cystathionine
DAP
Pyruvate
Threonine
α-Ketobutyrate
Hcy
Lysine
Isoleucine
Methionine
SMM
SAM
dcSAM
Spermidine
Spermine
Pyruvate
Alanine
Leucine
Valine
Acetyl-CoA
Oxaloacetate
Citrate
Isocitrate
Oxalosuccinate
α-Ketoglutarate
Succinate
Fumarate
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Glutamine
Citrulline
Histidine
Arginine
Putrescine

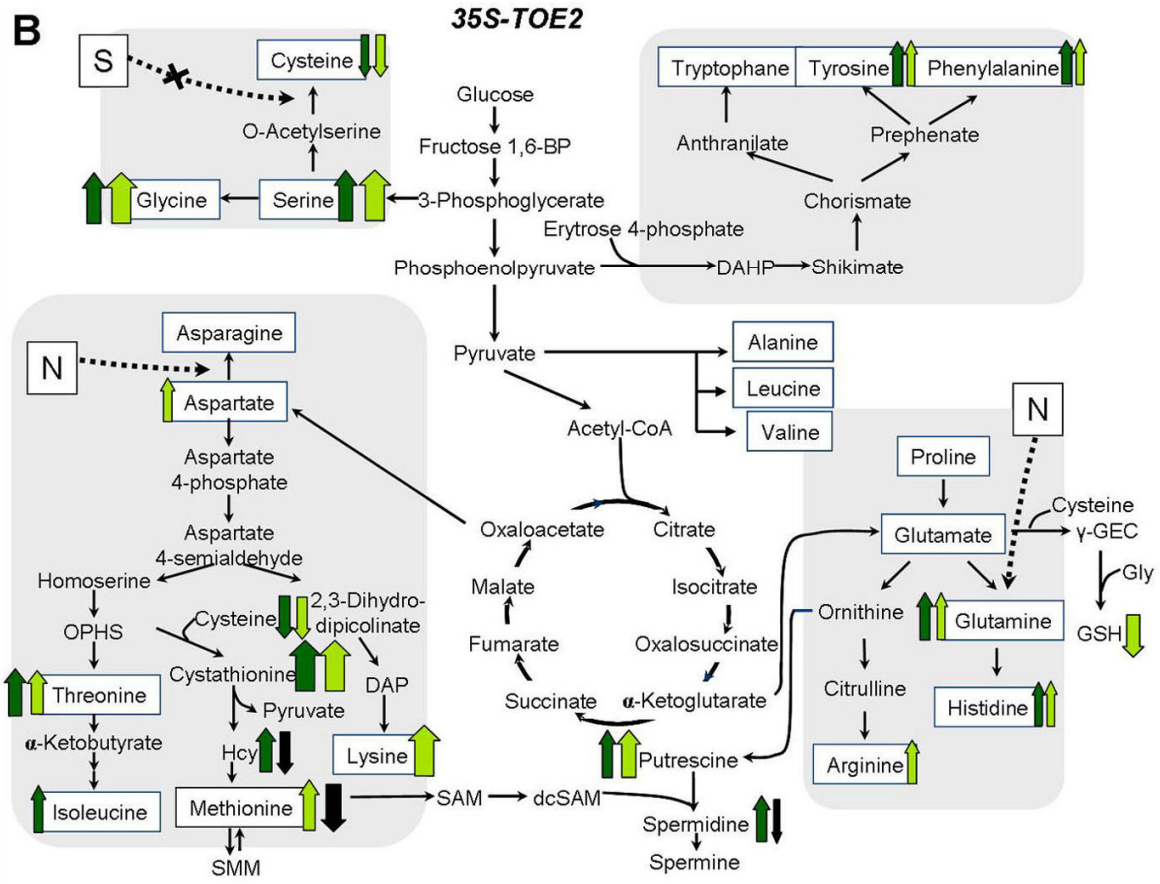
B
35S-TOE2
S
Cysteine
O-Acetylserine
Glycine
Serine
Glucose
Fructose 1,6-BP
3-Phosphoglycerate
Phosphoenolpyruvate
Erytrose 4-phosphate
DAHP
Shikimate
Chorismate
Anthranilate
Prephenate
Tryptophane
Tyrosine
Phenylalanine
N
Asparagine
Aspartate
Aspartate 4-phosphate
Aspartate 4-semialdehyde
Homoserine
OPHS
Cysteine
2,3-Dihydro-dipicolinate
Cystathionine
DAP
Pyruvate
Threonine
α-Ketobutyrate
Hcy
Lysine
Isoleucine
Methionine
SMM
SAM
dcSAM
Spermidine
Spermine
Pyruvate
Alanine
Leucine
Valine
Acetyl-CoA
Oxaloacetate
Citrate
Isocitrate
Oxalosuccinate
α-Ketoglutarate
Succinate
Fumarate
Malate
Proline
Glutamate
Cysteine
γ-GEC
Gly
GSH
Ornithine
Glutamine
Citrulline
Histidine
Arginine
Putrescine

Fig 4-3 Superposition of changes in metabolite pools on the amino acid biosynthetic pathways for leaves of *35S-TOE1* (**A**) and *35S-TOE2* (**B**) plants. The amino acids are grouped according to the biosynthetic families (*Biochemistry and Molecular Biology of Plants*, 2000). Dark red arrows indicate average changes in lines # 63, compared to wild type, and dark green arrows indicate changes in the line # 70, compared to wt, when plants were grown in S-sufficient conditions. Orange and pale green arrows indicate changes in S-deficient conditions, for both lines 63 and 70, respectively, compared to changes in wild type. Thickness of the arrow provides an indication on the strength of the observed changes and direction of the arrow indicates induction (up), or reduction (down) of the respective metabolite. Thickness of the orange and pale green arrows indicates, how the tendency of increase or decrease of respective metabolite is emphasized in respective 35S-AP2 lines, when compared to Col-0. Black arrows indicate the situation, when (under –S) the changes in 35S-AP2 plants are opposite than in wild type.

glycine, accumulation of putrescine, accumulation of nitrogen-rich amino acids (glutamine and asparagine) and increases of others amino acids, which result from carbon backbone allocations. Thus, similarities to the situation in S-starved wild type plants are obvious, with one exception, however. In wild type plants starved for sulphate, the synthesis of homocysteine is impaired through the depletion of cysteine, so the level of homocysteine also decreases. Upon a severe S-starvation stress this is often followed by a decrease in the level of methionine (as discussed previously, see chapter 4.A.3). According to the 'S-starvation-like' physiological phenotype of lines *35S-TOE1* and *35S-TOE2*, decreased levels of homocysteine and methionine would follow expectations. Interestingly, these metabolites are clearly increased in content in all 35S-AP2 lines grown in normal conditions (Fig. 4-3). Moreover, the highly elevated SMM level was additionally detected for the *35S-TOE1* line (Fig. 4-3A).

Results described above indicate strongly, that both lines, *35S-TOE1* and *35S-TOE2*, may posses a mechanism, which is promoting synthesis of methionine over synthesis of cysteine. This mechanism seems to promote *de-novo* synthesis of methionine rather than its recovery from the SMM pool, because the SMM level in *35S-TOE1* plants is increased as well. Although, this mechanism is presumably existing in wild type plants, in *35S-TOE1* and *35S-TOE2* overexpressors it is enhanced or amplified. As the amplification of the signal, which is promoting *de-novo* synthesis of methionine, is caused by overexpression of *TOE1* and *TOE2* genes, they might be a part of transcriptional regulation of methionine synthesis. According to obtained results, they would act as transcriptional inducers of the methionine biosynthesis pathway.

When sulphur deficiency stress was applied to the 35S-AP2 plants, the representation of the metabolites pools change dramatically. As mentioned above, the internal sulphate level in all 35S-AP2 overexpressors decreased even stronger than in starved wild type plants and this presumably caused more the pronounced decrease of cysteine. One can assume then, that 35S-AP2 plants suffer stronger from S-deficiency than wild type plants grown in the same S-limited conditions. Indeed, this hypothesis is confirmed by changes in metabolite pools, which in starved 35S-AP2 plants often exhibit the same tendency as in starved wild type plants, but this tendency is strongly pronounced (Fig. 4-3). Again, with one exception. Under S-limited conditions, levels of homocysteine and methionine decrease in *35S-TOE1* and *35S-TOE2* plants, and level of SMM decrease in the *35S-TOE1* plants, when compared to the levels of these metabolites in normal conditions. In contrast to that, in wild type plants levels of methionine and SMM are kept unchanged under S-limited conditions. Following this specific for 35S-AP2 overexpressors tendency, the levels of spermidine (and not putrescine!) also

decreased in this plants under –S. This proves, that the amplified in 35S-AP2 plants regulation mechanism is connected to the methionine biosynthesis, as methionine derivative, dcSAM, is essential for *de novo* spermidine sythesis.

Results obtained for *35S-TOE1* and *35S-TOE2* plants in S-deficient conditions suggest, that the mechanism proposed here of regulation of methionine biosynthesis is sensitive to and dependent on internal cysteine levels. In other words, the capacity of plant metabolism to maintain elevated methionine levels despite S-deficiency works only until a certain threshold. After a minimal and essential amount of internal cysteine is not available anymore, a breakdown of the regulatory mechanism is unavoidable, which was illustrated by the situation in S-depleted *35S-TOE1* and *35S-TOE2* plants.

4.B.2 Regulation of S-metabolism genes is realised *via* R1 domain

TOE1 and TOE2 transcription factors are suggested to work as flowering repressors (Aukerman and Sakai, 2003). Involvement of both factors in regulation of methionine synthesis might suggest connection of both these processes *via* ethylene production. This, however, could have been possible, if the non-flowering phenotype would have been exhibited exclusively by S-starved plants, which is not the case. Moreover, *35S-TOE2* plants flower slightly earlier than wild type plants. It is then worth considering, whether flowering processes and S-metabolism are controlled by the same regulatory mechanism, and, whether TOE1 and TOE2 have overlapping functions, a it was proposed by Aukerman and Sakai (2003).

Table 4-1 Morphological phenotypes of *TOE1* and *TOE2* transgenic lines studied to date.

Genotype	*TOE1*	*TOE2*
35S	late flowering[1,2]	early flowering[1]
KO	early flowering[2]	no effect on flowering[2]
Double mutant	Strong early flowering phenotype[2]	

Table legend:
[1] this study
[2] Aukerman and Sakai (2003)

Both *At2g28550* (*TOE1*) and *At5g60120* (*TOE2*) genes belong to the AP2-like subfamily, however, sequence analysis revealed the presence of two AP2 domains (R1 and R2) in the *TOE1* gene, while the *TOE2* gene possess only one AP2 domain (R1) (Fig. 1-10). Taking together these facts in conjunction with morphological phenotypes of *TOE1* and *TOE2* transgenic lines (Table 4-1), we propose two following hypothesis, explaining the mechanism in which the transcriptional regulation of methionine synthesis might be realised in plants (Figure 4-4 A and B).

The first hypothesis assumes, that the regulation of S-metabolism genes is presumably realised *via* interaction of R1 domains (common for both TOE1 and TOE2) with unknown *cis*-elements (X) characteristic for genes involved in S-metabolism or at least those involved in methionine biosynthesis (Fig 4-4A). The R2 domain, present only in TOE1 factor, is presumably responsible for

interactions with *cis*-elements (Y) typical for genes taking part in regulation of flowering processes. Thus, independent involvement of both TOE1 and TOE2 in regulation of methionine synthesis would be allowed by their R1 domains, while direct involvement of TOE2 in flowering would not be possible, unless realised *via* other conserved domains (yet unknown). Involvement of such domains (i.e. responsible for protein-protein interactions), which would explain the phenotype of the double mutant, *toe1-2 toe2-1* (Aukerman and Sakai, 2003), is conceivable.

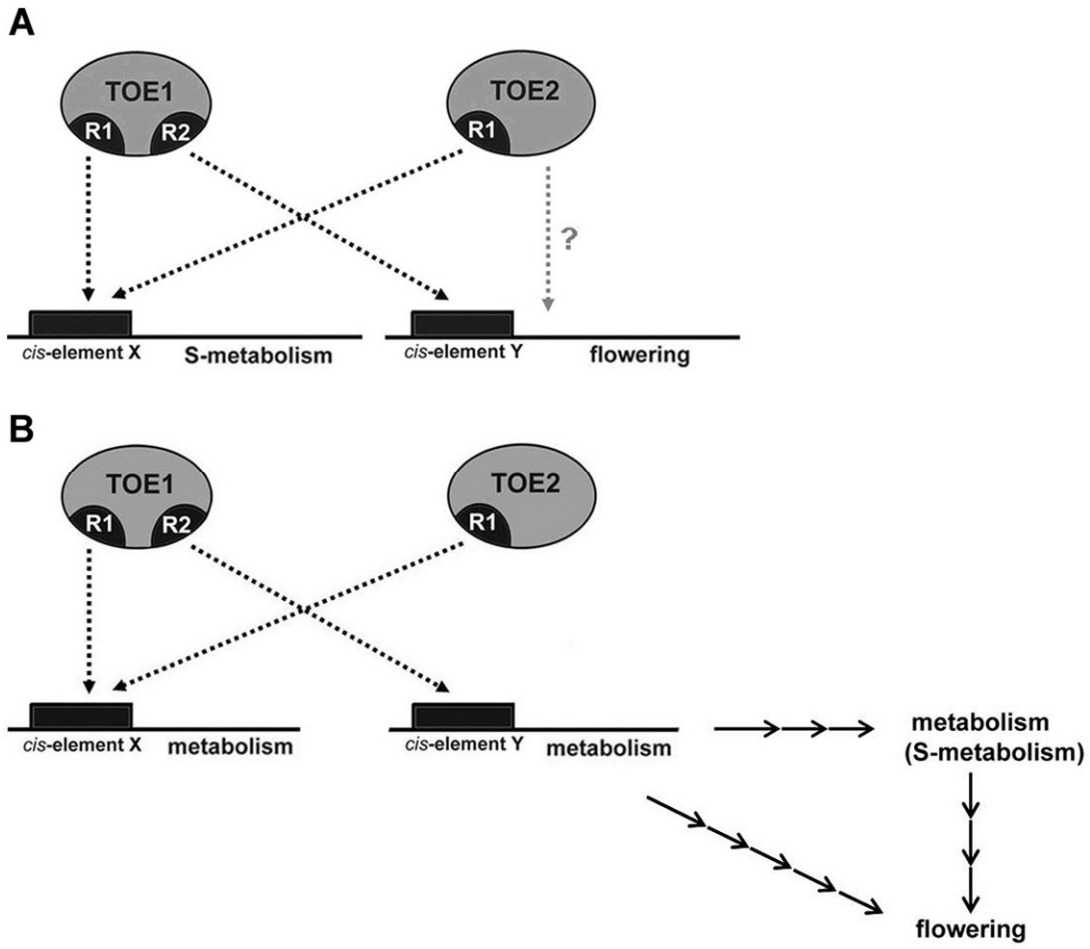

Fig 4-4 Two speculative models describing the regulation of expression of genes involved in metabolism/S-metabolism and flowering – proposed basing on results obtained in this study and by Aukerman and Sakai (2003). In the first model, the regulation of S-metabolism genes is presumably realised *via* interaction of R1 domains (common for both TOE1 and TOE2) with unknown *cis*-elements (X) characteristic for genes involved in S-metabolism or at least those involved in methionine biosynthesis. R2 domain, present only in TOE1 factor, is presumably responsible for interactions with *cis*-elements (Y) typical for genes taking part in regulation of flowering processes. Presence of other interactions involved in flowering regulation of TOE2 remains unknown (grey arrow) **(A)**. The second model assumes interaction of domains R1 and R2 with unknown *cis*-elements: X and Y, however, both types of *cis*-elements are presumably involved in the regulation of genes from different parts of metabolism, including S-metabolism/ biosynthesis of methionine. None of domains interact directly with *cis*-elements typical for genes involved in flowering processes. In this case, phenotypical changes in the flowering time, observed in *TOE1* and *TOE2* transgenic lines, are just a visible, pleiotropic consequence of various metabolic changes **(B)**. Black arrows indicate interactions between TF and respective *cis*-elements proposed in the hypothesis drawn in this work.

In the alternative model, domains R1 and R2 also interact with unknown *cis*-elements: X and Y, respectively. However, according to this hypothesis, both types of *cis*-elements are presumably involved in the regulation of genes from different parts of metabolism, including S-metabolism or at least biosynthesis of methionine (Fig 4-4B). None of domains interact directly with *cis*-elements typical for genes involved in flowering processes. In this case, phenotypical changes in the flowering time, observed in *TOE1* and *TOE2* transgenic lines (Table 4-1), are just a visible consequence of metabolic changes. Various and presumably distinct metabolic changes, caused by changes in either the *TOE1* or the *TOE2* gene expression, influence differently the plant phenotype, manifesting in a wide range of 'flowering-phenotypes' (Table 4-1). Nevertheless, both genes, *TOE1* and *TOE2*, are equally involved in the regulation of S-metabolism/methionine biosynthesis, as overexpression of each of those genes caused similar changes in levels of methionine-related metabolites (Fig. 4-3). Thus, according to the second hypothesis, the 'flowering phenotype' is only a pleiotropic effect of various metabolic changes caused by *TOE1* or *TOE2* overexpression.

Location of the *TOE1* and *TOE2* genes in the response network and the variety of function represented by their closest neighbours suggest that *TOE1* and *TOE2* genes are not directly involved in the first line of defence against sulphur starvation stress, rather might be involved in secondary responses to changed nutritional, oxidative etc. internal conditions (Fig. 3-32). As discussed before, it is very likely that TFs are involved in a complex multifactorial regulatory network (Broun, 2004). Response network analysis suggest that *TOE1* and *TOE2* genes may act as transcriptionally regulated, superior factors regulating other transcription factors directly involved in the regulation of S-metabolism genes, which would support the model of regulation created in the previous section (4.A.7). These direct regulators might be among those 8 TF genes, which were found to be the closest *TOE1* neighbours (see appendix D) or in any of the higher class of the network. They could simply have not passed the selection criteria for network reconstruction, or, finally, were not overexpressed in S-limited conditions, as discussed before (chapter 4.A.1 and 4.A.7).

5 SUMMARY AND CONCLUSIONS

Sulphur, a macronutrient essential for plant growth, is among the most versatile elements in living organisms. Unfortunately, little is known about regulation of sulphate uptake and assimilation by plants. This project aimed at identifying and characterising transcription factors that control sulphate metabolism in *Arabidopsis thaliana* and may prove useful in the future to improve sulphur-use efficiency in agriculture. To achieve that goal we postulated that factors regulating *Arabidopsis* responses to inorganic sulphate deficiency change their transcriptional levels under sulphur-limited conditions.

Two approaches were followed to identify transcription factors that control sulphate metabolism. In the first one, the short term S-starvation approach, young *Arabidopsis* seedlings were cultivated axenically, starved for sulphate for 48 hours and re-supplied with SO_4^{2-}. In the second, long term S-depletion approach, *Arabidopsis* plants were subjected to 10 and 13 days of constitutive and 6 to 10 days of induced sulphate starvation (Nikiforova et al., 2003).

In the short term S-starvation approach, a unique resource for studying the expression of TF genes in *Arabidopsis* (Czechowski et al., 2004), was used to identify TF genes potentially involved in sulphate signalling. The *Arabidopsis* qRT-PCR platform together with ATH1 chip hybridisation studies was successfully used to identify 20 TF genes which responded at the transcriptional level to S-deprivation. Finally, after cross-checking their sulphur-specificity, we selected a set of 8 genes that represents possibly specific and strong responses to sulphate deprivation: *AtWRKY43*, *At2g38340*, *AtWRKY24*, *At1g72570*, *AtbHLH052*, *WRKY93*, *ATAF1* and *At4g33960*. Most of these genes have not been characterized previously.

A loss of function approach was taken for functional characterisation of selected S-regulated TF genes. At least two different homozygous knock out lines were obtained for six candidate TF genes. Lack of the transcript or at least strong repression of transcript signal was detected for three out of six selected mutant lines. Thus, these lines, targeting *AtWRKY24*, *WRKY93* and *At4g33960*, were used for further preliminary characterisation. None of the homozygous KO lines showed visible aberrant phenotypes when grown on soil, or when screened for germination ratio, flowering time and root architecture changes. Thus, to demonstrate involvement of selected transcription factors in sulphur assimilation, axenically grown plants were subjected to sulphur starvation. Although levels of internal sulphate strongly decreased in all lines, neither mutant nor wild type lines showed any known visible indications of sulphur starvation compared to respective control plants, which had not been exposed to this stress.

Target expression analysis on SALK T-DNA insertion lines using quantitative RT-PCR revealed increased transcripts levels of many genes from sulphate uptake and assimilation. Expression pattern of these genes suggests that TF genes investigated in this approach act as their repressors. Especially *WRKY24* and *MYB93*, when impaired, seem to stimulate induction of many genes from S-metabolism. First mechanism of this regulation proposes that *WRKY24* and *MYB93* could act as the positive regulators of *SULTR1;3*, as it is the only gene down-regulated in these

mutants lines. Elevated transcript abundancies of all other genes acting downstream in the S assimilation pathway would result from the diminished sulphate uptake.

Another model of regulation assumes that TFs of interest are involved in complex multifactorial regulatory network. In this regulatory network, WRKY24 and MYB93 would act as superior factors regulating other transcription factors directly involved in the regulation of S-metabolism genes, such as the SLIM1/EIL3 factor (Maruyama-Nakashita et al., 2006). These direct regulators would act as inducers, while secondary regulators, WRKY24 and MYB93, would have a function as repressor. Thus, disruption of the *WRKY24* and *MYB93* gene function would cause induction of the primary factors, which could in turn induce target genes from sulphate uptake and assimilation. In wild type plants, a balance between repressors and inducers would maintain the expression of genes from sulphate metabolism at the steady-state level and allow to adjust it to the changes in internal sulphate concentration. According to the proposed model of regulation, regulators positioned more upstream in the hierarchy of interactions are commonly regulated at their expressional level by changes of sulphate conditions, while direct regulators are transcriptionally insensitive to the changes of sulphate availability, following their indispensability in the regulation mechanism. Hence, interactions between repressors and inducers probably does not occur at the expressional level. Despite similar influences of WRKY24 and MYB93 on expression of S-metabolism genes, suggested by transcript studies of respective knock out lines, differences in metabolite levels (especially thiols) observed between *WRKY24* and *MYB93* mutants indicate that WRKY24 and MYB93 are not redundant. Thus, involvement of other regulatory mechanisms, which would act independently from WRKY24 and MYB93 and, therefore, were responsible for differences observed between mutants, is conceivable. As –S-responsive gene expression is critical for plant survival in the –S condition, it is reasonable that plants have evolved multiplex regulatory mechanisms to control gene expression.

Summarizing, WRKY24 and MYB93 were first identified, transcriptionally regulated superior factors presumably involved in a multifactorial network regulating the assimilatory sulphur metabolism in higher plants.

Study of the *At4g33960* knock out lines revealed a down-regulation of *APS reductase* family genes and *SULTR1;3* in these mutants. This would implement possible regulatory function of the *At4g33960* gene product, which could act as a direct inducer of the SULTR1;3 gene and genes of APS reductases. Although the molecular function of the *At4g33960* gene remains unknown, this challenging result may suggest, that a weak homology of the expressed protein encoded by the *At4g33960* gene to a MADS-box region allows this protein to work as an transcription factor.

Functional characterisation of S-induced APETALA2 TF genes, *TOE1* (*At2g28550*) and *TOE2* (*At5g60120*), selected in the long term sulphur-depletion experiment (Nikiforova et al., 2003), was a second approach of this study. A gain-of-function approach was taken, in which the *TOE1* and *TOE2* genes were overexpressed under a strong constitutive promoter, the cauliflower mosaic virus 35S promoter.

T2 lines containing the *35S-At2g28550* construct and lines containing the *35S-At5g60120* construct revealed from 3 to 13-fold overexpression of both genes in roots and leaves of respective lines. Both *35S-TOE1* and *35S-TOE2* lines showed visible phenotype alterations when grown on soil,

vertical plates, and in hydroponics. The majority of plants containing the *35S-At2g28550* construct were strongly delayed in flowering and showed large rosette leaves, while most of the plants containing the *35S-At5g60120* construct were flowering earlier than wild type and had smaller rosette leaves. Following the idea, that some phenotypical alterations, especially those connected to nutritional metabolism, might not appear unless particular stress conditions are applied, we subjected hydroponically cultivated *35S-TOE1* and *35S-TOE2* lines to sulphur starvation. After ten days in S-limited conditions, some *35S-TOE1* lines exhibited an over 40 times accumulation of anthocyanins and, especially the line 63-10, retardation in growth, when compared to starved Col-0 plants.

Some interesting observations were made with respect to sulphate and metabolite contents in 35S-AP2 plants. All *35S-TOE1* and *35S-TOE2* lines exhibited significantly lower sulphate levels in leaves, which suggests that the 'sulphur-deficiency-sensing' in those lines, when starved for sulphate, was stronger than it was in S-starved wild type plants. Cysteine levels were found to be lower in leaves of studied lines, compared to Col-0, when grown in normal conditions. Strong differences in the content of many other amino acids were also found between wild type plants and all four 35S-AP2 lines. In general, most of amino acids were increased in content in *35S-TOE1* and *35S-TOE2* lines, often in both leaves and roots. According to the 'S-starvation-like' physiological phenotype of lines *35S-TOE1* and *35S-TOE2*, decreased levels of homocysteine and methionine would follow expectations. Interestingly, these metabolites are clearly increased in content in all 35S-AP2 lines grown in normal conditions. Moreover, highly elevated SMM levels were additionally detected for the *35S-TOE1* line.

When the sulphur deficiency was applied, the representation of the metabolite pools changed dramatically, especially in 35S-AP2 plants, when compared to Col-0. This includes decreases in cysteine content, accumulation of serine and glycine, accumulation of putrescine, accumulation of nitrogen-rich amino acids (glutamine and asparagine) and increases of other amino acids, which presumably was induced by carbon backbone allocations. Surprisingly, only metabolites connected to the methionine synthesis were clearly decreased in content in 35S-AP2 lines starved for sulphate.

Thus, we postulate that *35S-TOE1* and *35S-TOE2* may posses a mechanism, which is promoting *de-novo* synthesis of methionine over synthesis of cysteine. Although, this mechanism is presumably existing in wild type plants, in *35S-TOE1* and *35S-TOE2* overexpressors it is enhanced or amplified. Thus we postulate that *TOE1* and *TOE2* genes might be a part of transcriptional regulation of methionine synthesis, presumably acting as its transcriptional inducers.

We proposed two working hypotheses explaining the mechanism in which the transcriptional regulation of methionine synthesis might be realised in plants. According to the first hypothesis, the regulation of S-metabolism genes is presumably realised *via* interaction of R1 domains (common for both TOE1 and TOE2) with unknown *cis*-elements (X) characteristic for genes involved in S-metabolism or at least those involved in methionine biosynthesis. The R2 domain, present only in TOE1 factor, is presumably responsible for interactions with *cis*-elements (Y) typical for genes taking part in regulation of flowering processes. In the alternative model, domains R1 and R2 also interact with unknown *cis*-elements: X and Y, however, both types of *cis*-elements are presumably involved in the regulation of genes from different parts of metabolism, including S-metabolism or at least

biosynthesis of methionine. None of domains interact directly with *cis*-elements typical for genes involved in flowering processes. Both genes, *TOE1* and *TOE2*, are equally involved in the regulation of S-metabolism/methionine biosynthesis, as overexpression of each of those genes caused similar changes in levels of methionine-related metabolites. In this case, phenotypical changes in the flowering time, observed in *TOE1* and *TOE2* transgenic lines, are just a visible consequence of metabolic changes. Various and presumably distinct metabolic changes, caused by changes in either the *TOE1* or the *TOE2* gene expression, influence differently the plant phenotype, manifesting in a wide range of 'flowering-phenotypes' which, nevertheless, are only a pleiotropic effect of these various metabolic changes.

Response network analysis suggests that *TOE1* and *TOE2* genes may be involved in a complex multifactorial regulatory network and act as transcriptionally regulated, superior factors regulating other transcription factors directly involved in the regulation of S-metabolism genes, which would support the model of regulation created in the previous approach.

6 FUTURE OUTLOOK

To ensure, whether the loss of gene function studied by T-DNA insertion mutant lines is responsible for observed changes in the physiological phenotype, mutants should be backcrossed to the wild type to remove any additional T-DNA insertions in other than investigated loci. As using knockout mutant lines has many limitations, especially, if none obvious phenotype alteration can be expected, it is decided to test all selected sulphur-sensitive TF candidate genes by using gain-of-function approaches, with both constitutive and inducible promoters. Therefore all these TF genes have been cloned. *Arabidopsis* plants have been transformed and first transformants have been selected. They are now being checked in the axenic culture system. Overexpressors are grown in parallel to the wild type plants under S-sufficient and S-limited conditions.

In case of 35S-AP2 overexpressors, more study is needed to prove the speculated model of regulation of methionine synthesis by TOE1 and TOE2. It is necessary to find key enzyme genes being regulated by these TF. Target expression analysis on 35S-AP2 lines using i.e. quantitative RT-PCR should be extended to at least genes involved in methionine synthesis and metabolism, as these genes were indicated in this study as the possible target of TOE1 and TOE2 genes. ATH1 chip hybridisation would obviously bring even more information about transcription profile of these lines. Expressional behaviour of genes revealed by network reconstructions as direct neighbours of TOE1 and TOE2 genes should be of special interest. *In silico* promoter analysis of genes involved in S-metabiolism (especially those from methionine synthesis) should be used to reveal the presence of common *cis*-elements, which would presumably be a target for TOE1 and TOE2 proteins. As there are also limitations to the overexpression strategy, such as a possibility for creating neomorphic and antimorphic alleles (Zhang, 2003), it would be useful to interpret the overexpression data in conjunction with other supporting data i.e. physiological and morphological phenotype of knockout mutants. Despite of many limitations of the knockout mutant approach, a possibility for functional substitution of TOE1 and TOE2 genes is minor, as no duplicated twin of any of these genes was found throughout the *Arabidopsis* genome. By using the knockout mutant approach we expect to obtain a contrary morphological, physiological and molecular phenotype, than the one found in overexpressor lines. Especially, methionine levels are expected to be lower in knock out plants, while cysteine amounts would probably be elevated, in comparison to wild type plants, as the mechanism postulated here, which is promoting *de-novo* synthesis of methionine over synthesis of cysteine, would be disturbed by the *TOE1/TOE2* knockout.

All selected TF candidates, either from sulphur starvation and re-supply approach or from long-term S-starvation approach, should also be subjected to more targeted analysis. Especially, those based on protein-DNA interactions are suggested, such as chromatin immunoprecipitation (ChIP) assay or electrophoretic mobility shift assay (EMSA).

APPENDIX A Metabolic profile of sulphur deficiency in liquid culture experiments. In most cases values represent the averages out of three independent measurements, for which the standard deviations were calculated (for some metabolites fewer than three measurements were determined). Presented are all data which were identified at least for one out of two experiments. FN, full nutrition; S, sulphate in medium; AV, average; SD, standard deviation; nd, not determined; nm, not measurable

Metabolite	1st Experiment								2nd Experiment							
	FN AV	- S AV	30' S AV	3h S AV	FN SD	- S SD	30' S SD	3h S SD	FN AV	- S AV	30' S AV	3h S AV	FN SD	- S SD	30' S SD	3h S SD
Glycerol (lipid fraction)	0,894	0,821	0,766	0,775	0,049	0,048	0,040	0,116	1,167	0,910	1,034	1,025	0,072	0,088	0,090	0,141
C16:0	0,868	0,860	0,885	0,877	0,040	0,145	0,004	0,121	1,069	1,047	1,012	1,051	0,138	0,036	0,106	0,072
C18:cis[9,12]2	0,860	0,837	0,822	0,905	0,120	0,084	0,176	0,216	1,235	1,150	1,092	1,119	0,163	0,021	0,057	0,060
C18:cis[9,12,15]3	0,700	0,642	0,565	0,729	0,092	0,053	0,111	0,181	1,090	0,974	0,938	0,966	0,166	0,031	0,082	0,085
C18:0	1,006	0,946	1,013	1,007	0,034	0,137	0,125	0,148	1,300	1,225	1,238	1,127	0,061	0,059	0,094	0,073
C30:0	0,877	1,034	1,068	1,075	0,194	0,069	0,146	0,106	1,043	0,850	0,828	0,871	0,223	0,171	0,056	0,112
beta-Sitosterol	0,806	0,966	0,899	0,984	0,018	0,073	0,004	0,025	0,696	0,707	0,712	0,740	0,068	0,023	0,094	0,085
2-Hydroxy-Palmitic acid	1,002	1,219	1,159	1,208	0,046	0,173	0,116	0,050	0,804	0,855	0,919	0,895	0,060	0,046	0,072	0,130
Glycerophosphat (lipid fraction)	0,769	0,745	0,722	0,793	0,223	0,011	0,227	0,162	1,153	0,906	0,848	0,993	0,094	0,042	0,178	0,100
Methylgalactopyranosid	0,963	0,792	0,698	0,776	0,076	0,136	0,039	0,100	1,217	0,744	1,115	1,110	0,262	0,210	0,165	0,190
Hexadecadienoic Acid (C16:2)	1,277	0,520	0,669	0,835	0,275	0,213	0,116	0,014	nd	0,824	1,491	0,884	nm	0,191	0,169	0,104
Hexadecatrienoic Acid (C16:3)	0,686	0,381	0,264	0,446	0,135	0,135	0,066	0,059	1,131	0,868	0,976	1,056	0,151	0,080	0,039	0,167
C24:0	0,722	1,182	0,980	1,410	0,092	0,172	0,061	0,032	0,510	0,591	0,539	0,682	0,046	0,069	0,040	0,037
Campesterol	0,953	1,177	1,114	1,240	0,064	0,050	0,110	0,080	0,929	0,886	0,909	0,906	0,006	0,030	0,132	0,095
C17:0	0,836	0,914	0,989	1,011	0,121	0,198	0,115	0,001	1,129	1,120	1,113	1,102	0,395	0,057	0,117	0,173
C20:1	1,201	1,254	1,020	1,358	0,116	0,245	0,115	0,409	0,972	1,012	0,931	0,869	0,450	0,207	0,420	0,173
Nervonic Acid (C24:1)	0,901	1,084	0,930	1,061	0,048	0,185	0,023	0,087	0,466	0,720	0,670	0,757	0,232	0,010	0,128	0,040
Pyruvate	0,827	0,973	0,745	0,912	0,210	0,175	0,116	0,155	1,113	0,774	0,803	0,862	0,224	0,052	0,043	0,134
Ala	5,192	9,429	8,730	9,971	1,497	2,481	3,376	4,333	7,676	10,188	10,506	9,136	2,518	0,717	1,793	0,896
Gly	1,361	10,517	8,392	8,343	0,324	0,397	1,705	1,718	1,698	5,672	6,488	5,620	0,014	1,103	0,817	1,367
Thr	1,817	2,671	2,406	2,954	0,355	0,284	0,332	0,495	2,395	2,698	2,711	2,527	0,248	0,205	0,419	0,125
Ser	1,269	9,302	7,142	6,954	0,295	1,087	0,255	1,250	2,571	7,129	7,306	6,211	0,234	1,034	1,729	1,032
Val	1,605	2,489	2,546	1,895	0,355	0,351	0,408	0,244	1,960	2,572	2,398	1,614	0,154	0,228	0,378	0,140
Asp	2,638	1,521	1,931	1,682	0,543	0,065	0,165	0,029	1,851	2,182	2,486	2,220	0,204	0,352	0,240	0,205
Phe	0,987	0,952	0,974	0,598	0,341	0,388	0,246	0,126	0,989	0,982	0,936	0,709	0,172	0,096	0,128	0,022

Ile	1,253	1,263	1,327	0,728	0,284	0,264	0,180	0,013	1,686	1,569	1,440	0,880	0,047	0,182	0,151	0,049
Leu	1,267	1,199	1,288	0,619	0,452	0,563	0,038	0,144	1,521	1,158	1,069	0,593	0,138	0,171	0,081	0,021
Methionine	1,460	1,197	0,921	0,697	0,431	0,300	nm	nm	0,912	0,514	0,441	0,363	0,012	0,145	0,088	0,053
Tryptophan	0,957	5,750	4,486	3,571	0,185	0,517	0,533	nm	1,597	3,483	3,336	2,376	0,305	0,483	0,514	0,099
Proline	1,384	1,826	1,578	1,568	0,066	0,336	0,048	nm	1,859	2,130	1,567	1,551	0,586	0,216	0,422	0,177
Arginine	4,161	2,400	1,272	2,555	0,933	0,003	0,067	nm	3,214	4,845	3,854	3,536	0,881	0,000	0,242	0,294
Tyrosine	1,489	2,178	2,223	1,489	0,719	0,539	0,079	0,041	nd	nd	nd	nd	nm	nm	nm	nm
Glutamine	6,375	11,681	11,127	11,575	2,024	0,261	0,131	nm	5,971	15,617	14,938	14,259	1,039	1,659	0,934	0,566
Glutamic acid	2,775	1,970	1,732	1,638	0,795	0,147	0,841	nm	2,411	2,899	2,464	2,409	0,229	0,138	0,146	0,256
Citrulline	3,934	5,642	4,917	30,079	2,852	1,001	0,022	nm	3,321	8,203	7,344	7,447	0,986	0,513	0,822	1,246
Putrescin	1,081	23,493	30,803	27,310	0,776	6,161	9,901	nm	0,997	14,339	16,478	11,833	0,023	2,485	0,114	1,048
Myo-Inositol	0,812	1,142	1,170	1,008	0,209	0,337	0,253	0,068	0,325	0,385	0,419	0,417	0,041	0,030	0,064	0,040
Sucrose	0,173	0,718	0,151	0,347	0,139	1,154	0,055	0,410	0,519	0,353	0,374	0,246	0,079	0,035	0,076	0,012
Fructose	0,379	0,688	0,540	0,490	0,041	0,453	0,099	0,299	0,462	0,276	0,281	0,315	0,083	0,024	0,020	0,099
Glucose	0,383	0,390	0,324	0,261	0,095	0,173	0,036	0,272	0,199	nd	0,095	nd	0,011	nm	nm	nm
Raffinose	0,095	0,246	0,392	0,335	0,071	0,196	0,333	nm	0,202	0,293	0,188	0,247	0,048	0,077	0,061	0,015
Ferulic acid	0,554	0,673	0,691	0,679	0,037	0,021	0,019	nm	0,270	0,290	0,255	0,311	0,042	0,052	0,050	0,021
Succinate	0,697	1,001	0,654	0,749	0,083	0,119	0,115	nm	0,669	0,522	0,479	0,471	0,081	0,077	0,039	0,047
Fumarate	0,530	0,383	0,350	0,349	0,031	0,008	0,003	nm	0,885	0,434	0,382	0,476	0,179	0,086	0,020	0,188
Malate	0,447	0,420	0,398	0,406	0,054	0,038	0,021	nm	0,892	0,399	0,377	0,390	0,183	0,050	0,035	0,068
Glycerol-3-phosphate (polar fraction)	0,694	1,222	1,488	1,769	0,148	0,389	0,103	nm	0,653	0,600	0,524	0,548	0,055	0,052	0,094	0,031
UDPGlucose	0,731	0,433	0,497	0,982	0,346	0,028	0,302	nm	0,913	nd	0,510	0,563	0,103	nm	0,070	0,056
Isopentenyl Pyrophosphate	0,268	0,461	0,583	0,596	0,169	0,246	0,388	nm	0,776	0,696	0,702	0,721	0,061	0,077	0,025	0,068
Glycerol (polar fraction)	1,108	1,096	1,307	0,779	0,159	0,380	0,279	0,169	1,100	1,071	1,029	1,047	0,031	0,056	0,049	0,046
Phosphate	0,606	0,959	1,105	1,239	0,055	0,275	0,193	0,056	0,535	0,565	0,687	0,708	0,009	0,134	0,087	0,166
GABA	3,489	4,070	2,987	3,326	0,352	1,771	0,374	0,246	3,246	3,964	3,345	3,354	0,262	0,366	0,507	0,383
Homoserine	9,874	5,284	4,067	6,561	2,998	nm	0,695	nm	nd	nd	nd	nd	nm	nm	nm	nm
Glyceric acid	0,069	0,407	0,466	0,530	0,039	0,041	0,045	0,012	0,228	0,338	0,367	0,370	0,093	0,058	0,103	0,045
Sinapic Acid	0,826	0,867	0,528	0,762	0,207	0,242	0,072	0,164	1,207	0,779	0,924	1,004	0,151	0,129	0,097	0,189
Shikimic Acid	0,389	0,357	0,332	0,385	0,023	0,043	0,019	0,005	0,418	0,321	0,344	0,398	nm	0,028	0,016	0,027
DOPA	0,409	1,478	1,398	1,609	0,063	0,162	0,367	0,178	nd	nd	nd	3,587	nm	nm	nm	0,009
Anhydroglucose	0,492	1,382	0,660	0,718	0,155	nm	0,064	0,341	1,056	0,864	0,846	0,702	0,189	0,042	0,225	0,112

Gluconic acid	0,409	0,563	0,495	0,552	0,197	0,123	nm	0,049	nd	nd	nd	nd	nm	nm	nm	nm
Ribonic acid	0,628	0,824	0,818	0,749	0,052	0,134	0,049	0,011	0,849	0,728	0,660	0,568	0,077	0,103	0,113	0,064
Beta-apo-8' carotenal	0,166	0,469	0,489	0,662	0,056	0,163	0,030	0,051	0,200	0,238	0,292	0,408	0,031	0,075	0,003	0,076
Beta-Carotene	0,536	0,219	0,481	0,483	0,296	0,178	0,152	0,564	0,762	0,815	0,767	0,885	0,156	0,106	0,151	0,035
Cryptoxanthin	0,825	0,396	0,406	0,854	0,308	0,095	0,101	0,607	1,329	1,056	1,162	1,385	0,123	0,118	0,142	0,082
Lutein	0,742	0,548	0,530	0,659	0,166	0,058	0,046	0,106	1,038	0,951	1,005	1,086	0,034	0,073	0,080	0,107
Zeaxanthin	0,690	0,413	0,454	0,686	0,142	0,039	0,057	0,265	1,032	0,883	1,004	1,045	0,071	0,048	0,082	0,107
Ubichinone-45 (Coenzyme Q9)	0,750	0,814	0,855	0,871	0,050	0,161	0,068	0,162	0,942	1,068	0,970	0,979	0,123	0,057	0,172	0,115
Coenzyme Q10	0,789	1,345	1,514	1,767	0,073	0,204	0,472	0,315	1,344	1,618	1,317	1,156	0,343	0,090	0,431	0,032
2,3 Dimethyl-5-phytylquinol	0,214	3,113	0,646	2,597	0,154	5,043	0,010	2,055	0,403	0,949	0,629	0,624	0,001	0,595	0,213	0,088
gamma-Tocopherol	0,220	2,801	0,774	3,303	0,139	4,464	0,088	2,887	0,390	0,918	0,547	0,582	0,021	0,732	0,051	0,125
alpha-Tocopherol	0,565	0,786	0,736	2,099	0,295	0,619	0,376	0,027	0,995	1,257	1,098	0,994	0,198	0,363	0,195	0,025

APPENDIX B Relative expression level of all sulphur responsive genes, which were up- or down-regulated more than 10 times (10<R<0,1) under S-deficient conditions in at least one experimental replica. Genes, which expression was changed more than 10 times under S-deficient conditions in both experimental replicas are depicted in bold. FN, full nutrition; S, sulphate in medium

		1st Experiment			2nd Experiment				
Gene ID	**Affymetrix spot ID**	**S starv vs FN**	**30' vs S starv**	**3h vs S starv**	**S starv vs FN**	**30' vs S starv**	**3h vs S starv**	**Gene description**	**Functional category**
At5g23010	249866_at	0,03	0,59	11,50	0,04	1,24	15,52	2-isopropylmalate synthase-like	amino acid metab.Alw-Val-Leu synthesis
At1g15380	262603_at	0,06	1,01	2,49	0,32	1,24	2,71	hypothetical protein	amino acid metab.aspartate family.Thr.degrad.
At3g19710	257021_at	0,02	1,10	14,58	0,02	1,08	19,16	branched-chain aa aminotransferase, put.	amino acid metab.branched chain aa aminotransf.
At3g08860	258983_at	153,06	0,75	0,05	36,79	0,70	0,06	putative aminotransferase	amino acid metab.central aa metab.Ala synth
At4g34710	253203_at	31,13	0,93	0,31	15,13	0,88	0,34	arginine decarboxylase SPE2	amino acid metab.glutamate family.Arg degrad
At3g03630	259172_at	0,10	0,19	2,54	0,65	0,72	2,07	O-acetylserine (thiol) lyase	amino acid metab.Cys synthesis
At4g24000	254189_at	25,80	2,09	1,54	1,30	0,82	0,80	AtCslG2 putative protein	cell wall.cellulose synthesis
At5g57550	247866_at	10,83	1,28	0,64	0,68	1,73	0,70	endoxyloglucan transferase	cell wall.modification
At3g48360	252367_at	0,01	3,83	12,67	1,32	0,82	1,69	putative protein MEL-26	cell.division
At5g19470	246038_s_at	12,14	0,79	0,12	2,47	1,02	0,40	thiamin pyrophosphokinase, putative	Co-factor and vitamine metabolism
At2g23060	267250_at	29,31	0,69	0,46	8,81	0,46	0,22	similar to hookless1 (HLS1)	development.unspecified
At5g25260	246927_s_at	4,00	7,42	0,58	0,10	1,86	2,46	nodulin - like protein	development.unspecified
At4g33070	253416_at	19,15	1,69	1,56	0,51	3,65	2,23	pyruvate decarboxylase-1 (Pdc1)	fermentation
At5g04120	245689_at	10,57	0,76	0,53	1,61	0,83	0,40	phosphoglycerate mutase - like protein	glycolysis
At2g21210	264014_at	0,01	5,86	6,57	1,05	1,17	1,57	putative auxin-regulated protein	hormone metabolism.auxin.induced
At1g34310	262566_at	0,07	3,71	3,57	0,19	1,58	2,73	auxin response factor 1, putative	hormone metabolism.auxin.signal transd
At1g05680	263231_at	28,98	0,70	1,51	5,42	1,33	4,27	put. indole-3-acetate beta-glucosyltransferase	hormone metabolism.auxin.synth-degrad
At4g15490	245352_at	11,60	0,94	0,69	2,07	1,10	0,84	indole-3-acetate betaglucosyltransferase like prot	hormone metabolism.auxin.synth-degrad
At1g74670	260221_at	0,09	0,83	1,62	0,81	1,05	0,94	GAST1-like protein	hormone metabolism.gibberelin.induced
At1g19640	261150_at	29,57	1,05	0,56	4,24	1,02	0,78	floral nectary-specific protein	hormone metabolism.jasmonate.induced
At5g48180	248713_at	16,72	0,76	0,20	5,78	0,85	0,23	putative protein	hormone metabolism.jasmonate.induced
At1g17420	261037_at	16,46	1,71	0,50	1,31	0,63	0,65	lipoxygenase	hormone metabolism.jasmonate.synth-degrad
At1g76690	259875_s_at	12,06	0,73	0,31	6,02	0,81	0,48	12-oxophytodienoate reductase (OPR2)	hormone metabolism.jasmonate.synth-degrad
At4g34510	253240_at	53,00	0,90	0,78	8,50	1,08	0,67	ketoacyl-CoA synthase fatty acid elongase1, put.	lipid metabolism.FA synthesis and FA elongation
At1g04220	264318_at	13,15	0,90	0,63	4,68	1,31	0,74	putative beta-ketoacyl-CoA synthase	lipid metabolism.FA synthesis and FA elongation

At2g28630	263443_at	0,10	2,84	2,95	0,82	1,22	1,60	putative fatty acid elongase	lipid metabolism.FA synthesis and FA elongation
At1g74460	260234_at	12,21	0,90	1,04	5,11	1,22	1,17	putative lipase/acylhydrolase	lipid metabolism.lipid degrad.GDSL-motif lipase
At4g38560	252977_at	15,00	1,03	0,34	0,73	1,21	0,82	phospholipase like protein	lipid metabolism.lipid degrad.lysophospholipases
At4g02280	255521_at	16,67	0,86	0,81	1,62	1,20	1,02	putative sucrose synthetase	major CHO metabolism.degradation.sucrose
At1g32900	261191_at	13,56	1,49	0,49	2,43	1,33	0,83	starch synthase, putative	major CHO metabolism.synthesis.starch
At2g26020	257365_x_at	0,19	0,32	0,68	0,05	0,60	1,15	putative antifungal protein	metal handling.binding, chelation and storage
At5g44420	249052_at	0,09	0,71	2,43	0,06	1,11	1,79	antifungal protein-like (PDF1.2)	metal handling.binding, chelation and storage
At1g19610	261135_at	0,06	0,40	0,30	0,19	1,03	0,80	defensin AMP1, putative	metal handling.binding, chelation and storage
At1g35910	256319_at	51,06	0,65	0,32	2,43	0,82	0,38	trehalose-phosphatase, putative	minor CHO metabolism.trehalose
At1g70290	264339_at	0,09	2,59	2,24	0,44	0,81	1,68	trehalose-6-phosphate synthase, putative	minor CHO metabolism.trehalose
At2g37770	267168_at	16,97	1,10	1,51	2,34	1,15	1,18	putative alcohol dehydrogenase	misc.alcohol dehydrogenases
At3g28740	256589_at	148,91	0,52	0,83	11,33	0,88	1,28	cytochrome P450, putative	misc.cytochrome P450
At3g26830	258277_at	86,46	0,95	0,31	0,45	1,23	0,77	putative cytochrome P450	misc.cytochrome P450
At5g36110	249684_s_at	39,50	0,99	1,15	1,27	0,79	0,96	cytochrome P450-like	misc.cytochrome P450
At5g57220	247949_at	24,71	0,85	0,53	0,57	1,58	1,48	cytochrome P450	misc.cytochrome P450
At5g23190	249881_at	19,52	0,88	0,78	7,24	1,25	0,95	cytochrome P450-like protein	misc.cytochrome P450
At1g19630	261134_at	19,14	1,11	0,14	3,60	0,88	0,04	cytochrome P450, putative	misc.cytochrome P450
At2g22330	264052_at	0,06	1,22	13,50	0,16	1,43	11,91	putative cytochrome P450	misc.cytochrome P450
At1g16410	262717_s_at	0,06	0,39	9,57	0,05	1,41	15,36	putative cytochrome P450	misc.cytochrome P450
At4g13770	254687_at	0,03	1,05	9,70	0,06	1,20	8,80	cytochrome P450 monooxygenase	misc.cytochrome P450
At3g60140	251428_at	527,41	0,74	0,03	341,44	0,71	0,01	beta-glucosidase-like protein	misc.gluco-, galacto- and mannosidases
At2g44460	267389_at	363,51	0,75	0,03	551,89	0,77	0,01	putative beta-glucosidase	misc.gluco-, galacto- and mannosidases
At1g02850	262118_at	12,45	0,95	1,00	1,93	1,03	1,25	beta-glucosidase, putative	misc.gluco-, galacto- and mannosidases
At1g17170	262518_at	28,27	0,71	1,02	4,48	1,28	1,86	putative glutathione transferase	misc.glutathione S transferases
At2g29460	266267_at	19,54	1,19	0,51	1,06	0,98	0,90	putative glutathione S-transferase	misc.glutathione S transferases
At5g17220	250083_at	16,27	1,64	1,10	3,55	1,13	1,43	glutathione S-transferase-like protein	misc.glutathione S transferases
At1g78340	260803_at	14,17	0,69	0,52	2,90	0,96	0,65	glutathione transferase, putative	misc.glutathione S transferases
At1g17180	262517_at	11,31	1,06	1,14	1,57	1,09	1,94	putative glutathione transferase	misc.glutathione S transferases
At2g29490	266290_at	10,31	0,96	1,00	3,92	0,93	1,26	putative glutathione S-transferase	misc.glutathione S transferases
At1g75960	262698_at	0,09	4,40	4,10	1,72	0,79	1,05	AMP-binding protein, putative	misc.misc2
At3g22740	258322_at	0,09	0,24	0,63	0,09	1,06	3,02	putative selenocysteine methyltransferase	misc.misc2
At1g26380	261021_at	25,00	0,49	0,03	1,06	1,12	0,68	hypothetical protein	misc.nitrilases, nitrile lyases

At4g20820	254430_at	11,00	0,70	0,08	17,71	0,44	0,17	reticuline oxidase - like protein	misc.nitrilases, nitrile lyases
At1g75290	256450_at	90,50	0,54	0,08	18,47	0,68	0,08	NADPH oxidoreductase, putative	misc.oxidases - copper, flavone etc.
At1g62540	265122_at	42,25	1,73	1,18	1,19	1,31	1,35	similar to flavin-binding monooxygenase-like prot.	misc.oxidases - copper, flavone etc.
At5g37980	249601_at	12,96	0,81	0,27	8,21	0,66	0,22	quinone oxidoreductase -like protein	misc.oxidases - copper, flavone etc.
At1g31680	246573_at	0,06	0,82	1,91	0,22	0,12	1,16	copper amine oxidase, putative	misc.oxidases - copper, flavone etc.
At1g65860	261913_at	0,03	3,18	18,88	0,12	0,88	6,78	flavin-containing monooxygenase FMO3, put.	misc.oxidases - copper, flavone etc.
At1g68850	260035_at	11,91	1,11	0,94	3,43	1,18	1,17	peroxidase ATP23a	misc.peroxidases
At2g35380	266625_at	11,75	0,69	0,93	4,27	1,28	1,78	putative peroxidase	misc.peroxidases
At2g15490	265501_at	40,60	1,06	0,93	9,60	1,17	1,80	putative glucosyltransferase	misc.UDP glucosyl and glucoronyl transferases
At3g46660	252487_at	32,18	0,75	0,32	1,80	1,44	1,70	UDP-glucose glucosyltransferase	misc.UDP glucosyl and glucoronyl transferases
At4g14090	245624_at	14,72	1,58	0,92	3,05	0,72	0,97	glucosyltransferase like protein	misc.UDP glucosyl and glucoronyl transferases
At2g20800	265422_at	26,00	1,46	2,12	1,86	0,35	6,35	putative NADH-ubiquinone oxireductase	mitochondrial electron transport / ATP synthesis
At5g02780	250983_at	113,22	0,94	0,22	2,93	1,12	0,43	putative protein	not assigned.no ontology
At4g21990	254343_at	29,04	0,35	0,06	10,79	0,25	0,10	PRH26 protein	not assigned.no ontology
At4g24140	254202_at	27,33	0,94	1,03	2,17	1,52	2,04	putative protein	not assigned.no ontology
At5g09530	250500_at	18,63	0,97	0,99	7,27	1,06	1,02	periaxin - like protein	not assigned.no ontology
At5g37990	249599_at	18,15	0,95	0,52	4,19	1,09	0,91	putative protein AtPP protein	not assigned.no ontology
At4g25440	254069_at	15,81	0,37	0,35	3,26	0,93	0,54	putative protein	not assigned.no ontology
At1g44060	259452_at	15,75	0,06	0,05	1,13	0,89	1,04	En/Spm-like transposon protein	not assigned.no ontology
At5g37970	249598_at	15,73	0,60	0,25	3,05	1,41	0,58	putative protein AtPP protein	not assigned.no ontology
At5g09520	250541_at	15,41	0,68	0,54	6,58	1,21	0,96	putative proline-rich protein	not assigned.no ontology
At2g41380	266368_at	14,74	1,10	0,87	1,97	1,24	0,80	putative embryo-abundant protein	not assigned.no ontology
At4g33330	253379_at	13,62	0,37	0,87	1,17	1,02	1,13	protein glycogenin glucosyltransferase, put.	not assigned.no ontology
At5g09480	245889_at	12,26	0,93	0,79	2,49	1,37	1,96	PEE-rich protein	not assigned.no ontology
At3g60420	251400_at	1,00	0,73	0,64	0,03	1,18	1,31	putative protein	not assigned.no ontology
At1g32540	256469_at	0,10	1,43	2,57	0,75	0,91	1,58	zinc-finger protein, putative	not assigned.no ontology
At5g45680	248962_at	0,09	1,08	1,53	0,56	0,86	1,10	putative protein	not assigned.no ontology
At1g49200	260770_at	0,08	0,58	3,79	0,73	0,75	1,40	RING-H2 finger protein RHA3a, putative	not assigned.no ontology
At3g15570	257294_at	0,05	1,10	1,81	0,52	1,50	0,83	non-phototropic hypocotyl protein, putative	not assigned.no ontology
At5g48850	248676_at	818,53	0,40	0,07	161,77	0,29	0,05	putative protein	not assigned.unknown
At3g49580	252269_at	427,54	0,47	0,08	105,83	0,38	0,12	putative protein	not assigned.unknown
At1g78990	257428_at	97,62	1,16	0,78	6,96	1,28	1,35	hypothetical protein	not assigned.unknown

At2g43390	260535_at	85,17	0,84	0,64	12,73	1,25	0,98	hypothetical protein	not assigned.unknown
At2g19200	267437_at	68,25	0,74	0,95	2,10	0,99	1,43	hypothetical protein	not assigned.unknown
At1g12030	257421_at	65,96	0,19	0,16	123,20	0,14	0,07	hypothetical protein	not assigned.unknown
At1g73120	262373_at	62,69	0,52	0,68	2,79	1,19	0,78	hypothetical protein	not assigned.unknown
At2g18660	266070_at	53,89	0,93	0,45	0,13	1,61	1,03	hypothetical protein	not assigned.unknown
At4g01870	255543_at	52,59	0,81	1,38	5,26	1,68	3,50	predicted protein	not assigned.unknown
At4g39670	252908_at	49,00	0,97	0,88	0,95	0,72	0,97	putative protein	not assigned.unknown
At1g03495	265091_s_at	47,38	1,19	0,90	3,64	1,11	0,83	hypothetical protein	not assigned.unknown
At5g24660	249752_at	46,72	0,33	0,13	21,93	0,34	0,10	putative protein	not assigned.unknown
At2g41730	260522_x_at	44,20	1,37	2,61	1,32	1,14	5,37	hypothetical protein	not assigned.unknown
At3g07390	259018_at	42,33	2,56	1,30	1,00	1,27	0,77	unknown protein	not assigned.unknown
At1g63820	260315_at	38,33	0,95	0,63	1,65	0,85	0,73	hypothetical protein	not assigned.unknown
At3g52710	252000_at	38,00	2,83	1,07	2,50	1,22	0,80	hypothetical protein	not assigned.unknown
At4g31330	253525_at	35,74	0,61	0,24	21,15	0,49	0,21	predicted protein	not assigned.unknown
At5g20790	246001_at	34,75	0,14	0,09	0,69	1,24	1,65	putative protein	not assigned.unknown
At2g15360	263561_at	28,00	0,04	0,04	28,00	0,39	0,50	unknown protein	not assigned.unknown
At5g04000	250882_at	25,57	0,47	0,30	1,88	0,70	0,57	putative protein	not assigned.unknown
At5g58780	247797_at	24,55	0,76	0,70	1,83	1,30	1,01	dehydrodolichyl diphosphate synthase	not assigned.unknown
At2g24470	265687_s_at	23,50	1,48	0,77	1,44	1,34	0,69	hypothetical protein	not assigned.unknown
At2g04070	263401_at	22,53	0,91	0,82	0,62	1,07	1,08	hypothetical protein	not assigned.unknown
At1g65730	262925_at	21,67	2,03	0,71	0,83	1,11	0,71	hypothetical protein	not assigned.unknown
At2g39650	267623_at	20,44	0,99	0,48	2,73	0,68	0,64	unknown protein	not assigned.unknown
At5g44550	249061_at	19,89	1,34	1,21	3,09	1,46	1,96	putative protein	not assigned.unknown
At1g04770	261177_at	17,55	0,18	0,08	9,53	0,19	0,11	hypothetical protein	not assigned.unknown
At4g26950	253940_at	17,38	1,12	0,38	0,78	1,12	0,58	putative protein	not assigned.unknown
At1g55990	262097_at	17,29	0,32	0,36	3,57	1,18	1,25	hypothetical protein	not assigned.unknown
At2g47950	266486_at	16,32	0,68	0,75	0,88	0,88	1,04	hypothetical protein	not assigned.unknown
At1g19960	261221_at	15,09	0,90	0,61	0,07	1,30	1,03	hypothetical protein	not assigned.unknown
At5g52760	248322_at	14,88	0,09	0,08	0,08	0,88	1,77	putative protein	not assigned.unknown
At1g03700	264842_at	14,41	0,59	0,71	10,88	0,88	0,90	hypothetical protein	not assigned.unknown
At1g63340	265105_s_at	14,38	0,84	0,65	1,82	1,49	1,42	unknown protein	not assigned.unknown
At1g56660	245677_at	13,71	1,83	0,83	1,44	1,26	0,73	hypothetical protein	not assigned.unknown

At2g22510	263998_at	13,19	1,40	0,93	3,82	1,24	1,21	unknown protein	not assigned.unknown
At1g21240	259559_at	12,50	0,12	0,36	0,06	2,82	3,00	wall-associated kinase 3, hypoth.	not assigned.unknown
At3g27150	256750_at	12,10	0,63	0,35	5,73	0,82	0,32	unknown protein	not assigned.unknown
At2g39030	266142_at	12,01	0,84	0,26	0,92	0,93	0,58	unknown protein	not assigned.unknown
At1g04000	265039_at	11,83	0,88	0,64	1,21	1,22	0,71	unknown protein	not assigned.unknown
At3g28580	256989_at	10,79	1,83	0,50	0,26	1,53	1,09	hypothetical protein	not assigned.unknown
At3g22435	258458_at	10,78	1,33	0,76	1,09	0,89	0,87	expressed protein	not assigned.unknown
At3g49820	252251_at	10,23	0,33	0,68	1,89	0,94	0,68	hypothetical protein	not assigned.unknown
At4g38080	253024_at	10,09	1,02	0,83	5,35	1,15	1,32	putative protein	not assigned.unknown
At3g22231	256766_at	4,65	0,19	0,18	0,02	1,13	1,53	expressed protein	not assigned.unknown
At3g48640	252345_at	3,00	0,56	0,22	0,05	2,56	1,11	hypothetical protein	not assigned.unknown
At2g14560	265837_at	1,17	1,77	1,22	0,07	0,94	1,22	unknown protein	not assigned.unknown
At4g01390	255621_at	1,05	1,18	0,73	15,77	0,97	0,75	hypothetical protein	not assigned.unknown
At5g22555	249893_at	0,90	0,67	0,89	0,08	1,00	0,89	expressed protein	not assigned.unknown
At1g50930	256208_at	0,60	0,33	1,17	0,04	11,50	13,75	hypothetical protein	not assigned.unknown
At5g62330	247476_at	0,42	0,82	0,82	0,06	0,85	0,80	putative protein	not assigned.unknown
At2g25510	265611_at	0,22	0,77	0,69	0,09	0,95	1,32	unknown protein	not assigned.unknown
At5g03350	250942_at	0,17	3,58	3,13	0,04	1,93	3,47	putative protein	not assigned.unknown
At3g28120	257308_at	0,11	0,67	1,33	14,50	1,14	0,07	unknown protein	not assigned.unknown
At2g32880	267644_s_at	0,09	2,34	1,50	0,58	1,35	1,21	unknown protein	not assigned.unknown
At3g15450	258402_at	0,09	2,16	1,47	0,66	0,65	1,98	unknown protein	not assigned.unknown
At2g16005	263098_at	0,09	1,67	1,13	0,48	0,66	0,57	expressed protein	not assigned.unknown
At1g33055	261567_at	0,08	0,25	0,84	0,55	0,93	1,76	expressed protein	not assigned.unknown
At4g16980	245459_at	0,08	1,06	2,29	0,70	1,21	1,08	hypothetical protein	not assigned.unknown
At5g66550	247102_at	0,07	4,69	2,48	1,08	0,74	0,72	putative protein	not assigned.unknown
At2g02200	266113_x_at	0,07	0,78	1,44	7,10	0,66	0,62	hypothetical protein	not assigned.unknown
At3g16670	258419_at	0,07	0,88	0,70	0,35	0,52	0,66	unknown protein	not assigned.unknown
At3g05730	258897_at	0,07	0,88	0,85	0,38	0,71	0,77	unknown protein	not assigned.unknown
At1g23390	262986_at	0,07	1,16	3,06	0,27	0,83	2,26	unknown protein	not assigned.unknown
At4g12970	254794_at	0,06	0,35	2,65	0,77	0,85	1,10	putative protein	not assigned.unknown
At3g22210	256796_at	0,06	2,03	1,41	0,56	0,69	1,09	hypothetical protein	not assigned.unknown
At2g20480	263369_at	0,06	1,24	11,82	0,44	1,16	2,12	unknown protein	not assigned.unknown

At1g15045	260741_at	0,05	1,30	0,78	0,14	1,08	1,55	hypothetical protein	not assigned.unknown
At1g19530	260668_at	0,05	1,58	2,15	0,69	0,86	1,04	unknown protein	not assigned.unknown
At5g34885	246641_s_at	0,05	0,75	0,83	1,80	0,82	1,19	Expressed protein	not assigned.unknown
At2g25680	265897_at	0,04	1,72	13,00	0,36	0,93	2,61	hypothetical protein	not assigned.unknown
At5g55450	248062_at	0,03	0,88	1,00	0,05	2,78	4,69	unknown protein	not assigned.unknown
At2g04790	263674_at	0,03	2,58	1,17	0,80	0,94	1,08	hypothetical protein	not assigned.unknown
At2g14140	263280_x_at	0,03	3,40	0,60	5,09	1,46	1,55	hypothetical protein	not assigned.unknown
At1g73870	260380_at	0,02	8,83	1,17	0,47	2,39	1,72	hypothetical protein	not assigned.unknown
At5g10210	250472_at	0,02	1,43	1,57	0,32	1,51	2,51	putative protein	not assigned.unknown
At1g32830	261234_x_at	0,02	1,33	1,00	1,14	1,14	1,36	hypothetical protein	not assigned.unknown
At3g05770	258739_s_at	0,00	3,00	3,00	0,03	13,60	15,00	unknown protein	not assigned.unknown
At4g39675	252882_at	0,00	1,00	5,00	0,50	0,50	1,49	Expressed protein	not assigned.unknown
At1g73290	260091_at	33,67	0,99	1,00	9,56	1,03	0,72	putative serine carboxypeptidase	protein.degradation
At5g50260	248545_at	19,30	1,35	0,77	1,66	1,24	1,02	cysteine proteinase	protein.degradation
At2g22980	267265_at	0,09	0,67	0,75	0,81	0,66	0,45	putative serine carboxypeptidase I	protein.degradation
At3g08870	258982_at	42,71	0,53	0,13	2,60	0,54	0,28	putative serine/threonine protein kinase	protein.postranslational modification
At1g68690	262228_at	15,53	2,27	0,63	1,54	1,42	0,57	protein kinase,	protein.postranslational modification
At3g25250	257840_at	12,00	0,74	0,93	1,21	1,07	2,17	protein kinase	protein.postranslational modification
At1g36370	260126_at	228,09	0,82	0,06	19,85	0,37	0,06	putative hydroxymethyltransferase	PS.photorepiration
At4g09010	255078_at	0,09	1,25	1,23	0,71	0,70	0,83	putative protein ascorbate peroxidase	redox.ascorbate and glutathione
At1g32350	260706_at	38,89	0,77	0,05	0,57	1,16	0,57	oxidase, putative	redox.dismutases and catalases
At1g03020	263168_at	24,75	0,89	0,17	14,69	0,50	0,13	putative glutaredoxin	redox.glutaredoxins
At3g62930	251195_at	0,06	0,54	4,46	0,28	2,76	4,90	glutaredoxin -like protein	redox.glutaredoxins
At5g18600	249996_at	0,04	0,54	4,88	0,30	1,13	4,80	glutaredoxin -like protein	redox.glutaredoxins
At4g15700	245506_at	0,02	0,22	3,89	0,26	1,84	4,11	glutaredoxin homolog	redox.glutaredoxins
At4g15680	245392_at	0,01	0,90	3,70	0,36	1,31	3,89	glutaredoxin	redox.glutaredoxins
At4g15660	245504_at	0,01	0,50	10,75	0,37	1,08	2,25	glutaredoxin	redox.glutaredoxins
At4g15690	245505_at	0,00	0,50	7,00	0,03	4,11	29,44	glutaredoxin	redox.glutaredoxins
At1g60740	264923_s_at	0,09	0,72	0,66	0,75	0,75	0,48	peroxiredoxin, putative	redox.periredoxins
At1g66390	260140_at	229,82	1,07	0,38	1,57	1,36	0,28	Myb-related transcription factor, putative	RNA.regulation of transcription
At2g46130	266597_at	42,38	0,55	0,85	2,62	1,13	1,28	putative WRKY-type DNA binding protein	RNA.regulation of transcription
At1g34670	262406_at	41,65	1,13	1,05	3,01	1,24	1,69	myb-related protein, putative	RNA.regulation of transcription

At5g06510	250688_at	21,07	1,82	1,01	1,70	1,04	0,74	transcription factor-like protein	RNA.regulation of transcription
At1g74650	260220_at	18,14	0,68	0,44	2,78	1,19	0,64	putative MYB family transcription factor	RNA.regulation of transcription
At5g16770	246477_at	13,71	1,00	0,66	3,12	0,85	0,65	putative transcription factor (MYB9)	RNA.regulation of transcription
At1g18750	261423_at	13,58	0,28	0,66	1,61	0,51	0,71	homeodomain transcription factor	RNA.regulation of transcription
At3g46070	252567_at	12,00	0,62	0,64	0,79	1,16	1,86	zinc finger -like protein; Zat7	RNA.regulation of transcription
At3g56400	251705_at	1,33	0,81	0,43	0,10	0,99	1,09	DNA-binding protein 4; WRKY4	RNA.regulation of transcription
At1g56650	245628_at	65,40	1,32	0,42	18,76	1,05	0,46	anthocyanin2, putative	secondary metabolism.flavonoids
At4g22870	254283_s_at	10,77	1,94	0,96	1,38	1,44	2,22	anthocyanidin synthase - like protein	secondary metabolism.flavonoids
At5g18660	250006_at	0,07	0,48	1,39	0,61	0,78	0,94	2 -hydroxyisoflavone reductase, put.	secondary metabolism.flavonoids
At1g18870	261428_at	68,29	0,51	0,07	22,41	0,87	0,16	isochorismate synthase, putative	secondary metabolism.phenylpropanoids
At1g33030	261216_at	46,50	1,15	0,67	0,87	0,93	0,93	catechol O-methyltransferase, putative	secondary metabolism.phenylpropanoids
At2g29130	266783_at	32,18	0,58	0,99	1,10	1,32	0,80	putative laccase (diphenol oxidase)	secondary metabolism.phenylpropanoids
At1g09500	264514_at	17,86	0,53	0,73	2,09	0,81	1,02	putative cinnamyl alcohol dehydrogenase	secondary metabolism.phenylpropanoids
At3g09580	258708_at	0,03	2,07	7,97	0,43	1,23	1,74	putative oxidoreductase	secondary metabolism.terpenoids
At3g01830	258947_at	79,67	0,70	0,17	0,68	1,22	0,34	hypothetical protein	signalling.calcium
At1g76640	259866_at	0,71	1,20	0,80	0,07	0,24	0,88	putative calmodulin	signalling.calcium
At1g51830	246375_at	20,33	1,64	0,06	8,71	1,93	1,23	light repressible receptor protein kinase, put.	signalling.receptor kinases
At2g24130	265992_at	11,64	0,12	1,31	1,57	0,83	0,95	putative receptor-like protein kinase	signalling.receptor kinases
At1g53540	260978_at	12,19	0,96	0,82	0,75	1,48	0,93	17.6 kDa heat shock protein	stress.abiotic
At2g38340	267026_at	82,67	0,43	0,03	11,75	4,49	0,53	DREB-like AP2 domain transcription factor	stress.abiotic.drought/salt
At2g41690	245107_at	29,33	1,59	0,61	0,88	1,14	1,27	putative heat shock transcription factor	stress.abiotic.heat
At2g26560	245038_at	13,61	0,99	0,54	0,30	2,01	1,60	putative protein	stress.abiotic.unspecified
At4g12830	254783_at	0,09	1,04	2,52	1,16	0,70	1,12	2-hydroxy-6-oxohepta-2,4-dienoate hydrolase	stress.abiotic.unspecified
At3g05950	258746_at	0,02	4,75	4,70	0,65	0,84	0,71	germin-like protein	stress.abiotic.unspecified
At3g24954	257592_at	71,75	1,17	0,92	0,80	0,85	1,06	leucine-rich repeat disease resistance protein,put	stress.biotic
At1g45616	245799_at	52,50	2,71	0,99	2,09	1,33	0,59	disease resistance protein, putative	stress.biotic
At4g13900	254741_s_at	20,80	0,83	0,92	0,24	1,39	1,35	putative disease resistance protein	stress.biotic
At1g33960	260116_at	0,67	0,87	1,71	0,07	0,93	1,40	AIG1 protein	stress.biotic
At1g23730	265170_at	90,51	0,89	0,13	62,44	0,87	0,10	putative carbonic anhydrase	TCA / org. transformation.carbonic anhydrases
At5g19410	246067_at	43,00	1,11	0,90	16,39	1,01	0,67	membrane transporter - like protein	transport misc
At1g66760	256324_at	24,06	1,22	0,13	13,18	0,86	0,16	MATE efflux family protein, putative	transport misc
At5g13580	250239_at	21,07	1,11	0,70	5,81	1,23	0,89	ABC transporter-like protein	transport.ABC transp and multidrug resist systems

At1g53270	260643_at	12,85	1,27	0,70	8,54	1,68	1,00	hypothetical protein	transport.ABC transp and multidrug resist systems
At3g55090	251824_at	12,57	0,56	0,58	6,10	1,30	0,87	ABC transporter - like protein	transport.ABC transp and multidrug resist systems
At3g53510	251944_at	10,98	0,70	0,63	4,93	1,13	0,95	ABC transporter -like protein	transport.ABC transp and multidrug resist systems
At3g56200	251722_at	12,32	0,60	0,19	7,94	0,50	0,13	putative protein	transport.amino acids and peptides
At4g35180	253181_at	11,22	1,08	0,59	0,27	1,61	1,35	amino acid permease - like protein	transport.amino acids and peptides
At5g18290	250025_at	11,61	0,53	0,15	11,38	0,56	0,13	putative protein	transport.Major Intrinsic Proteins.SIP
At1g61800	264400_at	33,18	2,98	0,57	2,21	1,80	0,68	glucose-6-phosphate-translocator precursor, put.	transport.metabolite transp. at envelope membr
At5g60770	247591_at	12,31	0,69	0,65	0,81	1,29	1,06	high-affinity nitrate transporter ACH1 - like prot.	transport.nitrate
At3g47950	252395_at	10,46	0,96	1,07	1,19	0,96	1,11	H+-transporting ATPase - like protein	transport.p- and v-ATPases
At4g21680	254396_at	15,38	1,09	0,26	2,21	0,94	0,34	peptide transporter - like protein	transport.peptides and oligopeptides
At4g08620	255105_at	24,67	1,25	0,35	29,30	0,93	0,34	putative sulfate transporter	transport.sulphate
At3g12520	256244_at	21,76	0,79	0,12	10,86	0,75	0,16	sulphate transporter, putative	transport.sulphate
At1g78000	262133_at	16,22	0,69	0,21	16,59	0,54	0,21	high affinity sulphate transporter, putative	transport.sulphate
At1g77990	262134_at	4,20	0,94	0,32	12,03	0,73	0,34	sulfate transporter, putative	transport.sulphate
At5g26220	246884_at	107,66	0,42	0,03	184,33	0,37	0,02	cation transport protein chaC, E. coli, putative	transport.unspecified cations
At3g05400	259133_at	113,59	1,09	0,06	28,89	0,78	0,06	sugar transporter, putative	transporter.sugars

APPENDIX C Quantitative RT-PCR results for TF genes, exhibiting more than 5-fold transcript changes, under sulphur deprivation, 30 minutes, and 3 hours after sulphate replenishment. FN, full nutrition; S, sulphate in medium; E, efficiency of reaction; Ct, threshold cycle

AGI	Gene name	Family or domain	Ampli-con (bp)	1+E	Ct				ΔCt				ΔΔCt		x-fold change		
					FN	-S	30 'S	3h S	FN	-S	30 'S	3h S	-S/FN	30'/-S	3h/-S	-S/FN	30'/-S	3h/-S
Up-regulated under S starvation																		
AT1G66390	AtMYB90	MYB	150	2,07	27,21	19,62	21,46	21,07	10,07	1,69	3,48	3,11	-8,38	1,78	1,42	443,51	0,27	0,36
AT1G56650	AtMYB75	MYB	103	1,74	28,23	19,81	35,80	21,12	11,09	1,88	17,82	3,16	-9,21	15,93	1,28	164,24	0,0001	0,49
AT1G66380	AtMYB114	MYB	150	1,80	31,82	24,62	28,39	26,57	14,68	6,69	10,40	8,61	-7,99	3,71	1,92	109,03	0,11	0,32
AT5G43410		AP2-EREBP	68	1,64	40,00	33,38	33,15	34,19	22,71	15,18	15,42	16,17	-7,53	0,24	0,99	41,53	0,89	0,61
AT1G72350		MADS	62	1,60	38,33	31,41	32,01	33,92	21,19	13,48	14,03	15,96	-7,71	0,55	2,48	37,17	0,77	0,31
AT2G46130	AtWRKY43	WRKY	109	1,80	28,19	23,78	23,13	23,74	10,81	5,36	5,32	5,56	-5,44	-0,04	0,19	24,84	1,02	0,89
AT2G17180		C2H2	78	1,65	39,14	34,12	32,65	33,85	21,85	15,93	14,91	15,83	-5,93	-1,01	-0,10	19,74	1,66	1,05
AT2G38340		AP2-EREBP	60	1,77	29,16	24,97	24,49	26,09	11,87	6,77	6,76	8,07	-5,10	-0,01	1,30	18,41	1,01	0,48
AT1G64000	AtWRKY56	WRKY	65	1,83	26,22	22,45	22,02	22,53	8,84	4,03	4,22	4,34	-4,81	0,19	0,31	18,26	0,89	0,83
AT3G02940	AtMYB107	MYB	144	1,89	27,72	23,96	26,07	24,87	10,58	6,03	8,09	6,91	-4,55	2,05	0,88	17,97	0,27	0,57
AT1G49190	ARR19	ARR B	112	1,51	40,00	33,92	40,00	37,41	22,86	15,99	22,02	19,45	-6,86	6,02	3,46	16,89	0,08	0,24
AT5G41570	AtWRKY24	WRKY	65	1,71	29,63	25,47	24,96	25,58	12,25	7,05	7,15	7,40	-5,19	0,10	0,34	16,00	0,95	0,83
AT1G72570		AP2 EREBP	93	1,49	37,72	31,77	31,96	30,99	20,43	13,57	14,23	12,98	-6,86	0,66	-0,59	15,39	0,77	1,27
AT5G38800	AtbZIP43	bZIP	63	1,75	32,34	28,36	27,75	28,08	15,05	10,16	10,02	10,06	-4,89	-0,15	-0,10	15,35	1,08	1,06
AT5G51860		MADS	70	1,58	40,00	35,07	40,00	34,63	22,63	16,77	23,41	17,18	-5,86	6,63	0,41	14,35	0,049	0,83
AT1G30670	AtbHLH052	bHLH	120	1,69	39,78	35,78	35,14	34,54	22,64	17,86	17,16	16,58	-4,78	-0,70	-1,27	12,14	1,44	1,95
AT4G13480	AtMYB79	MYB	131	1,61	34,04	29,77	29,48	28,60	16,90	11,85	11,49	10,64	-5,05	-0,35	-1,20	10,93	1,18	1,77
AT2G11990		MADS	82	1,75	36,33	33,14	32,80	34,29	18,95	14,72	15,00	16,10	-4,23	0,27	1,38	10,71	0,86	0,46
AT5G46830	AtbHLH028, ERF2	bHLH	94	1,64	36,31	32,30	35,38	34,70	19,17	14,38	17,40	16,74	-4,79	3,02	2,36	10,65	0,23	0,31
AT1G34670	AtMYB93	MYB	72	1,77	27,66	24,49	24,41	24,62	10,52	6,57	6,43	6,66	-3,95	-0,14	0,09	9,47	1,08	0,95
AT2G40350		AP2-EREBP	75	1,59	40,00	36,18	32,57	33,09	22,71	17,98	14,83	15,08	-4,73	-3,15	-2,90	9,06	4,33	3,86
AT2G13150	AtbZIP31	bZIP	60	1,78	33,64	30,73	27,97	28,21	16,35	12,54	10,24	10,20	-3,82	-2,30	-2,34	8,99	3,76	3,84
AT3G30210	AtMYB121	MYB	90	1,57	35,57	31,53	31,54	31,92	18,42	13,61	13,56	13,97	-4,82	-0,05	0,36	8,84	1,02	0,85
AT4G21440	AtMYB102	MYB	131	1,75	35,02	31,92	33,25	32,51	17,88	14,00	15,27	14,55	-3,88	1,27	0,55	8,73	0,49	0,73
AT2G33720		ARP	100	1,72	34,79	31,76	31,31	30,71	17,50	13,56	13,58	12,69	-3,94	0,02	-0,87	8,53	0,99	1,61
AT2G47520		AP2-EREBP	82	1,75	28,46	25,56	25,15	24,28	11,17	7,36	7,42	6,27	-3,81	0,06	-1,09	8,38	0,97	1,84

AT1G66550	AtWRKY67	WRKY	82	1,50	40,00	35,81	35,36	35,15	22,62	17,40	17,55	16,96	-5,23	0,16	-0,44	8,24	0,94	1,19
AT1G77450		NAC	92	1,70	28,16	25,22	24,25	26,41	10,78	6,80	6,45	8,22	-3,98	-0,35	1,43	8,20	1,20	0,47
AT1G17950	AtMYB52	MYB	123	1,77	28,13	25,24	26,23	25,42	10,99	7,31	8,24	7,46	-3,68	0,93	0,15	8,14	0,59	0,92
AT1G73410	AtMYB54	MYB	116	1,80	26,08	23,44	24,69	23,80	8,94	5,52	6,71	5,85	-3,42	1,19	0,33	7,43	0,50	0,82
AT3G12720	AtMYB67	MYB	94	1,76	27,48	24,72	24,54	24,15	10,34	6,80	6,56	6,20	-3,54	-0,24	-0,60	7,42	1,15	1,40
AT2G24430		NAC	98	1,78	27,55	25,17	24,12	24,88	10,17	6,75	6,31	6,69	-3,42	-0,44	-0,05	7,25	1,29	1,03
AT1G69560	AtMYB105	MYB	80	1,45	34,26	29,79	30,28	29,72	17,12	11,87	12,29	11,77	-5,25	0,43	-0,10	7,17	0,85	1,04
AT1G66370	AtMYB113	MYB	92	1,68	30,34	27,36	27,07	28,49	13,20	9,44	9,09	10,54	-3,77	-0,35	1,10	7,06	1,20	0,56
AT5G27960		MADS	93	1,43	39,64	34,91	40,00	35,47	22,49	16,99	22,02	17,51	-5,51	5,03	0,53	7,06	0,17	0,83
AT4G29930	AtbHLH027	bHLH	92	1,89	24,22	21,98	21,82	21,98	7,08	4,06	3,84	4,02	-3,03	-0,22	-0,04	6,92	1,15	1,02
AT3G26790	FUSCA3,FUS3	ABI3VP1	61	1,89	29,16	27,06	26,83	27,89	11,87	8,86	9,09	9,87	-3,01	0,23	1,01	6,82	0,86	0,52
AT1G01720	ATAF1	NAC	60	1,87	22,87	20,86	20,16	21,81	5,49	2,44	2,35	3,63	-3,05	-0,09	1,19	6,78	1,06	0,47
AT5G16770	AtMYB9	MYB	109	1,76	28,56	26,14	25,36	26,14	11,19	7,84	8,77	8,70	-3,34	0,92	0,86	6,62	0,59	0,62
AT3G46080		C2H2	61	1,74	30,04	27,47	27,15	28,60	12,60	9,19	9,28	10,43	-3,41	0,09	1,24	6,59	0,95	0,50
AT5G65230	AtMYB53	MYB	60	1,83	28,31	26,12	25,64	26,30	10,94	7,82	9,04	8,86	-3,11	1,22	1,04	6,59	0,48	0,53
AT2G47190	AtMYB2	MYB	99	1,77	27,26	24,76	25,99	25,13	10,12	6,83	8,01	7,17	-3,29	1,18	0,34	6,58	0,51	0,82
AT5G54070	HSFA9	HSF	93	1,46	40,00	36,08	40,00	40,00	22,62	17,66	22,20	21,81	-4,96	4,53	4,15	6,44	0,18	0,21
AT5G22570	AtWRKY38	WRKY	72	1,71	34,94	32,54	31,71	33,43	17,56	14,12	13,91	15,25	-3,44	-0,21	1,12	6,38	1,12	0,55
AT4G09820	AtbHLH042, TT8	bHLH	60	1,66	32,05	29,19	28,06	28,97	14,91	11,26	10,08	11,01	-3,65	-1,19	-0,25	6,38	1,83	1,14
AT5G65100		EIL	98	1,62	37,87	34,87	33,40	34,60	20,43	16,60	15,53	16,44	-3,83	-1,07	-0,16	6,30	1,67	1,08
AT1G25340	AtMYB116	MYB	119	1,77	36,75	34,31	34,97	40,00	19,61	16,38	16,99	22,04	-3,23	0,61	5,67	6,27	0,71	0,040
AT1G77950		MADS	75	1,90	31,05	29,05	29,03	30,40	13,91	11,12	11,05	12,44	-2,79	-0,07	1,32	6,02	1,05	0,43
AT5G06510		CCAAT-HAP2	96	1,81	24,41	22,24	21,28	23,21	6,97	3,96	3,40	5,05	-3,01	-0,56	1,08	5,99	1,39	0,52
AT5G41200		MADS	84	1,70	32,29	29,71	29,17	29,31	15,14	11,78	11,19	11,36	-3,36	-0,60	-0,43	5,97	1,37	1,25
AT5G38620		MADS	71	1,35	40,00	34,95	35,03	34,71	22,86	17,03	17,04	16,76	-5,83	0,02	-0,27	5,87	0,99	1,08
AT4G34410		AP2-EREBP	64	1,73	30,74	28,46	28,87	32,10	13,45	10,27	11,13	14,08	-3,19	0,87	3,82	5,75	0,62	0,12
AT5G01860		C2H2	89	1,69	32,44	29,97	29,47	29,11	15,00	11,69	11,60	10,95	-3,31	-0,09	-0,74	5,73	1,05	1,48
AT5G63790		NAC	112	1,87	22,26	20,53	19,79	21,02	4,89	2,11	1,99	2,83	-2,77	-0,12	0,72	5,69	1,08	0,64
AT4G36590	AGL40	MADS	83	1,81	32,52	30,60	31,56	32,02	15,14	12,31	14,96	14,58	-2,84	2,66	2,27	5,42	0,21	0,26
AT1G80840	AtWRKY40	WRKY	82	1,74	27,78	25,80	25,76	27,32	10,40	7,39	7,96	9,13	-3,01	0,57	1,74	5,28	0,73	0,38
AT1G18960		MYB	143	1,62	34,22	31,80	30,92	32,06	16,84	13,38	13,12	13,87	-3,46	-0,26	0,49	5,25	1,13	0,79
AT5G14010		C2H2	67	1,63	36,92	34,37	36,47	34,06	19,48	16,10	18,60	15,90	-3,38	2,50	-0,20	5,23	0,29	1,10

AT1G31630		MADS	66	1,56	40,00	37,12	40,00	40,00	22,86	19,19	22,02	22,04	-3,67	2,83	2,85	5,17	0,28	0,28
AT5G08790	ATAF-2	NAC	130	1,72	26,62	24,63	24,10	25,26	9,24	6,21	6,29	7,07	-3,02	0,08	0,86	5,16	0,96	0,63
AT1G27730	ZAT10	C2H2	72	1,80	25,09	23,23	24,02	25,01	7,81	5,03	6,28	6,99	-2,78	1,25	1,96	5,11	0,48	0,32
AT4G29230		NAC	85	1,71	29,56	27,57	26,81	27,73	12,18	9,16	9,00	9,54	-3,02	-0,15	0,39	5,07	1,08	0,81
AT2G30380		MYB-like	65	1,43	40,00	36,47	40,00	37,32	22,62	18,05	22,20	19,14	-4,57	4,15	1,09	5,06	0,23	0,68
Down-regulated under S starvation																		
AT1G68520	COL6	CO-like	72	1,89	21,06	24,13	23,87	23,31	3,62	5,85	6,00	5,15	2,23	0,14	-0,71	0,24	0,91	1,57
AT3G61950	AtbHLH067	bHLH	69	1,74	26,11	29,46	30,26	30,11	8,97	11,53	12,27	12,15	2,57	0,74	0,62	0,24	0,66	0,71
AT5G49450	AtbZIP1	bZIP	88	1,81	22,46	25,82	25,23	25,03	5,18	7,62	7,50	7,02	2,44	-0,12	-0,60	0,24	1,08	1,43
AT5G43290	AtWRKY49	WRKY	121	1,74	24,45	28,13	26,74	29,87	7,07	9,71	8,94	11,68	2,64	-0,77	1,97	0,23	1,53	0,34
AT3G25790		MYB-like	81	1,52	27,09	31,47	31,24	31,18	9,65	13,19	13,37	13,02	3,54	0,17	-0,17	0,23	0,93	1,07
AT1G22130		MADS	89	1,65	30,03	34,05	34,61	33,39	12,89	16,12	16,62	15,44	3,23	0,50	-0,69	0,20	0,78	1,41
AT3G56970	AtbHLH038	bHLH	140	1,64	26,60	30,79	34,44	27,18	9,46	12,86	16,46	9,23	3,41	3,59	-3,64	0,19	0,17	5,99
AT1G73870	COL7	CO-like	61	1,85	23,59	27,16	26,35	26,64	6,15	8,88	8,48	8,48	2,73	-0,40	-0,40	0,19	1,28	1,28
AT3G56980	AtbHLH039	bHLH	94	1,72	27,50	31,37	32,59	26,53	10,36	13,45	14,61	8,58	3,09	1,16	-4,87	0,19	0,53	14,20
AT2G23740		C2H2	134	1,46	34,04	39,55	34,14	33,51	16,76	21,35	16,41	15,49	4,59	-4,94	-5,86	0,18	6,47	9,14
AT3G23130	SUPERMAN,SUP	C2H2	99	1,70	31,14	35,46	36,14	34,38	13,70	17,18	18,27	16,21	3,48	1,09	-0,97	0,16	0,56	1,68
AT5G04150	AtbHLH101	bHLH	61	1,64	29,89	35,17	34,66	33,40	12,75	17,24	16,68	15,45	4,49	-0,56	-1,79	0,11	1,32	2,42
AT1G06180	AtMYB13	MYB	149	1,80	25,64	30,69	31,00	27,22	8,27	12,39	14,41	9,78	4,12	2,02	-2,61	0,09	0,31	4,62
Up-regulated after 30 minutes of S re-supply																		
AT5G45980		HB	124	1,68	40,00	40,00	35,34	40,00	22,56	21,72	17,46	21,84	-0,84	-4,26	0,11	1,54	9,15	0,94
Down-regulated after 30 minutes of S re-supply																		
AT2G07440		ARR	110	1,98	19,47	21,24	23,36	20,07	2,33	3,32	5,38	2,12	0,99	2,06	-1,20	0,51	0,24	2,27
AT2G27070	ARR13	ARR B	92	1,53	31,62	30,60	34,06	31,36	14,48	12,68	16,08	13,40	-1,80	3,40	0,73	2,15	0,24	0,73
AT2G41130	AtbHLH106	bHLH	149	1,73	25,88	25,93	28,66	26,09	8,74	8,01	10,68	8,14	-0,73	2,67	0,13	1,49	0,23	0,93
AT2G31220	AtbHLH010	bHLH	138	1,72	28,61	29,61	32,41	29,03	11,47	11,68	14,43	11,08	0,21	2,74	-0,60	0,89	0,23	1,39
AT5G02810	APRR7	ARR	64	1,89	23,39	24,18	26,62	24,61	6,25	6,26	8,64	6,65	0,01	2,38	0,40	1,00	0,22	0,78
AT5G58080	ARR18	ARR B	83	1,74	36,27	38,90	40,00	37,70	18,90	20,60	23,41	20,26	1,70	2,80	-0,34	0,39	0,21	1,20
AT4G25560	AtMYB18	MYB	150	1,69	27,92	27,23	30,35	27,71	10,78	9,30	12,37	9,76	-1,48	3,07	0,45	2,17	0,20	0,79
AT2G31180	AtMYB14	MYB	132	1,74	25,75	26,26	29,23	26,25	8,60	8,34	11,25	8,30	-0,27	2,91	-0,04	1,16	0,20	1,02
AT2G12900	AtbZIP33	bZIP	86	1,47	37,77	36,15	40,00	36,35	20,48	17,95	22,27	18,34	-2,53	4,31	0,38	2,64	0,19	0,86
AT4G00540	AtMYB3R2	MYB	83	1,44	34,64	35,34	40,00	36,93	17,50	17,42	22,02	18,98	-0,08	4,60	1,56	1,03	0,19	0,57

AT5G07700	AtMYB76	MYB	138	1,87	23,97	24,04	25,06	24,10	6,60	5,74	8,47	6,66	-0,86	2,73	0,92	1,71	0,18	0,56
AT3G56970	AtbHLH038	bHLH	140	1,64	26,60	30,79	34,44	27,18	9,46	12,86	16,46	9,23	3,41	3,59	-3,64	0,19	0,17	5,99
AT5G42640		C2H2	60	1,53	36,02	35,31	39,25	35,30	18,58	17,03	21,38	17,14	-1,55	4,35	0,11	1,92	0,16	0,95
AT3G45170		GATA	150	1,66	31,03	33,76	37,71	33,54	13,89	15,83	19,73	15,59	1,94	3,90	-0,25	0,37	0,14	1,13
AT1G56160	AtMYB72	MYB	94	1,71	33,98	33,58	37,34	33,30	16,84	15,65	19,36	15,34	-1,19	3,71	-0,31	1,89	0,14	1,18
AT3G05860		MADS	150	1,73	26,70	26,00	29,74	27,69	9,56	8,08	11,76	9,73	-1,48	3,68	1,66	2,25	0,13	0,40
AT2G26150	HSFA2	HSF	142	1,86	24,12	24,02	27,44	22,02	6,98	6,10	9,46	4,06	-0,88	3,36	-2,03	1,73	0,12	3,54
AT5G41580		ZnF	100	1,78	24,43	24,86	28,59	24,70	7,29	6,93	10,61	6,74	-0,35	3,67	-0,19	1,23	0,12	1,12
AT4G09460	AtMYB6	MYB	138	1,74	24,31	25,12	29,41	25,56	7,17	7,20	11,42	7,60	0,03	4,23	0,41	0,98	0,097	0,80
AT1G60250		CO-like	97	1,61	33,55	35,06	40,00	40,00	16,11	16,78	22,13	21,84	0,67	5,34	5,06	0,73	0,08	0,09
AT2G20180	PIL5, AtbHLH015	bHLH	60	1,52	32,84	33,67	40,00	34,64	15,70	15,74	22,02	16,68	0,04	6,27	0,94	0,98	0,07	0,67
AT5G39810		MADS	114	1,60	32,99	33,72	39,86	33,08	15,85	15,79	21,88	15,12	-0,06	6,09	-0,67	1,03	0,06	1,37
AT5G26650		MADS	85	1,55	35,54	32,68	40,00	33,95	18,40	14,75	22,02	15,99	-3,65	7,27	1,24	4,92	0,042	0,58
AT5G38860		bHLH	86	1,65	32,51	32,17	38,69	32,22	15,37	14,24	20,71	14,26	-1,13	6,47	0,02	1,76	0,039	0,99
AT1G65330	AGL37, PHE1	MADS	60	1,70	29,87	33,17	39,63	31,66	12,73	15,24	21,64	13,70	2,51	6,40	-1,54	0,26	0,033	2,26
Up-regulated after 3 hours of S re-supply																		
AT5G27880		C2H2	85	1,48	40,00	40,00	40,00	35,34	22,56	21,72	22,13	17,18	-0,84	0,40	-4,54	1,39	0,85	6,01
AT1G01030		ABI3VP1	84	1,46	40,00	40,00	37,75	35,30	22,71	21,80	20,01	17,29	-0,91	-1,79	-4,51	1,41	1,96	5,44
AT1G25470		AP2-EREBP	145	1,45	37,57	37,59	35,28	33,08	20,29	19,39	17,54	15,06	-0,89	-1,85	-4,33	1,39	1,99	5,02
Down-regulated after 3 hours of S re-supply																		
AT1G12610		AP2-EREBP	81	1,62	31,97	31,70	31,23	34,61	14,69	13,50	13,50	16,59	-1,18	-0,01	3,08	1,76	1,00	0,23
AT3G44460	AtbZIP67,DPBF2	bZIP	68	1,65	32,56	33,03	33,69	35,85	15,27	14,83	15,96	17,83	-0,44	1,12	3,00	1,24	0,57	0,22
AT5G51990		AP2-EREBP	60	1,79	33,09	31,45	31,52	34,09	15,80	13,25	13,79	16,08	-2,55	0,54	2,83	4,40	0,73	0,19
AT5G49240	APRR4	ARR B	84	1,66	34,33	34,93	34,62	38,22	17,19	17,00	16,64	20,26	-0,18	-0,37	3,26	1,10	1,20	0,19
AT4G00120	AtbHLH040	bHLH	79	1,68	33,39	34,38	33,99	37,64	16,24	16,45	16,01	19,68	0,20	-0,44	3,23	0,90	1,26	0,19
AT1G26310	AGL10,CAL1	MADS	65	1,53	33,09	35,75	35,15	40,00	15,95	17,83	17,17	22,04	1,88	-0,66	4,22	0,45	1,32	0,17
AT2G25230	AtMYB100	MYB	103	1,59	35,82	35,90	38,07	40,00	18,68	17,97	20,08	22,04	-0,70	2,11	4,07	1,39	0,37	0,15

APPENDIX D List of closest neighbors of two AP2 TF genes of interest which were present in the dataset used for network reconstruction. TF genes are marked with star (*).

Vertex name	Vertex# (of2014)	Pearson to gene	Class	Gene description	Gene category	1st Exp minS/FN	2nd Exp minS/FN
At2g28550 class 6 (85 NEIGHBOURS)							
At1g69370	1617	0,898	5	chorismate mutase, putative	amino acid metab.phenylalanine.synthesis	0,86	0,86
At1g15380	1788	0,881	5	hypothetical protein	amino acid metab.aspartate fam.threonine.degrad	0,06	0,32
At3g60740	1597	0,895	5	beta-tubulin cofactor - like protein	cell.organisation	1,73	0,88
At2g37450	1626	0,862	5	nodulin-like protein	development.unspecified	0,44	0,90
At1g66340*	1764	0,886	5	ethylene-response protein, ETR1	hormone metabolism.ethylene.signal transd	1,19	1,13
At3g60620	1596	0,875	5	phosphatidate cytidylyltransferase - like protein	lipid metabolism.phospholipid synthesis	0,77	1,05
At1g13110	1695	0,910	5	putative cytochrome P450 monooxygenase	misc.cytochrome P450	0,82	0,92
At5g12940	1558	0,872	5	putative protein	not assigned.no ontology	0,73	0,69
At4g24050	1865	0,861	5	putative protein	not assigned.no ontology	0,63	1,07
At3g28000	1427	0,875	5	hypothetical protein	not assigned.unknown	1,95	3,11
At1g61620	1675	0,868	5	hypothetical protein	not assigned.unknown	0,86	0,91
At5g23680	1612	0,848	5	putative protein	not assigned.unknown	1,45	1,56
At4g38340*	1620	0,881	5	putative protein	not assigned.unknown	3,75	0,67
At5g61670	1674	0,862	5	putative protein	not assigned.unknown	1,02	1,05
At3g20550	1505	0,898	5	unknown protein	not assigned.unknown	1,07	1,39
At3g27610	1546	0,895	5	unknown protein	not assigned.unknown	1,39	1,21
At1g67330	1708	0,850	5	unknown protein	not assigned.unknown	0,84	1,04
At1g68780	1848	0,889	5	unknown protein	not assigned.unknown	0,80	1,20
At2g35750	1867	0,918	5	unknown protein	not assigned.unknown	0,94	0,79
At1g77000	1604	0,913	5	F-box protein family, AtFBL5	protein.degradation	1,16	1,09
At5g23210	1630	0,848	5	serine carboxypeptidase II-like protein	protein.degradation	0,50	0,91
At4g26530	1701	0,881	5	fructose-bisphosphate aldolase - like protein	PS.calvin cyle	0,04	0,25
At1g09140	1605	0,855	5	putative SF2/ASF splicing modulator, Srp30	RNA.processing	1,72	1,30
At3g54810*	1727	0,939	5	putative protein GATA transcription factor 3	RNA.regulation of transcription	1,66	0,95
At2g48160	1658	0,878	5	unknown protein similar to transcription factor HUA2	RNA.regulation of transcription	2,14	1,28
At5g50780	1573	0,858	5	putative protein	signalling.misc	1,09	1,35

At5g24090	1598	0,897	5	acidic endochitinase	stress.biotic	3,20	1,71
At5g53130	1587	0,902	5	cyclic nucleotide-regulated ion channel	transport.cyclic nucl or calcium regul channels	1,08	1,04
At2g39450	1645	0,857	5	unknown protein	transport.metal	1,46	1,13
At5g10240	1315	0,864	6	asparagine synthetase ASN3	amino acid metab.aspartate fam.asparagine.synth	0,77	0,54
At4g32420	764	0,881	6	CDC28/cdc2-like kinase associating arg-ser cyclophilin, put	cell.cycle	1,30	1,11
At5g20110	1293	0,849	6	dynein light chain - like protein	cell.organisation	1,77	1,33
At5g15530	850	0,852	6	biotin carboxyl carrier protein precursor-like	Co-factor and vitamine metabolism	0,80	0,90
At5g47370*	1869	0,911	6	homeobox-leucine zipper protein-like	development.unspecified	1,72	0,87
At1g18800	1386	0,940	6	unknown protein	DNA.synthesis/chromatin structure	0,87	1,00
At3g16440	1843	0,915	6	putative lectin	hormone metabolism.jasmonate responsive	0,72	0,79
At1g13580	820	0,902	6	longevity assurance protein, putative	lipid metabolism.'exotics'. sphingolipids	0,79	0,93
At4g39170	1870	0,858	6	phosphatidylinositol-phosphatidylcholine transfer SEC14-like prot	lipid metabolism.lipid transfer proteins etc	1,33	1,06
At3g27660	1448	0,870	6	oleosin isoform	lipid metabolism.TAG synthesis	2,08	2,05
At4g26900	1779	0,908	6	glutamine amidotransferase/cyclase	misc.misc2	1,03	1,02
At2g29290	895	0,903	6	putative tropinone reductase	misc.nitrilases, troponine reductases	0,16	0,82
At3g43670	956	0,853	6	amine oxidase -like protein	misc.oxidases - copper, flavone etc.	0,96	1,37
At1g62560	1949	0,920	6	similar to flavin-containing monooxygenase	misc.oxidases - copper, flavone etc.	0,21	0,14
At1g05560	1450	0,853	6	UDP-glucose:indole-3-acetate beta-D-glucosyltransferase, put	misc.UDP glucosyl and glucoronyl transf	8,80	2,04
At4g23450	978	0,888	6	putative protein zinc finger protein	not assigned.no ontology	1,14	1,87
At3g02340	806	0,939	6	RING zinc-finger protein, putative	not assigned.no ontology	1,36	1,14
At5g07100*	821	0,869	6	SPF1-like protein	not assigned.no ontology	0,62	1,15
At2g06210	1300	0,886	6	putative nuclear phosphoprotein TPR repeat protein	not assigned.no ontology	0,91	0,98
At2g19620	1024	0,866	6	putative SF21 protein	not assigned.no ontology	0,65	1,03
At5g04040	1256	0,861	6	putative protein	not assigned.unknown	1,47	1,14
At4g32780	1456	0,874	6	putative protein	not assigned.unknown	13,19	1,98
At3g60200	1063	0,855	6	putative protein	not assigned.unknown	1,18	0,92
At1g27340	1384	0,873	6	unknown protein	not assigned.unknown	1,79	1,10
At4g36940	809	0,858	6	hypothetical protein	not assigned.unknown	0,76	0,77
At4g15750	854	0,908	6	hypothetical protein	not assigned.unknown	0,85	1,35
At1g54090	1306	0,898	6	hypothetical protein	not assigned.unknown	1,41	1,12
At4g04340	798	0,879	6	predicted protein of unknown function	not assigned.unknown	0,75	1,18
At5g54470*	754	0,869	6	putative protein	not assigned.unknown	3,67	1,21

At5g46190	1888	0,880	6	putative protein	not assigned.unknown	1,17	1,27
At1g47310	927	0,916	6	unknown protein	not assigned.unknown	1,13	1,09
At3g21080	932	0,868	6	unknown protein	not assigned.unknown	0,32	0,17
At3g21810	1030	0,907	6	unknown protein	not assigned.unknown	1,08	0,89
At1g10690	1116	0,878	6	unknown protein	not assigned.unknown	4,09	0,38
At1g49320	1148	0,855	6	unknown protein	not assigned.unknown	1,14	1,45
At1g80040	1309	0,932	6	unknown protein	not assigned.unknown	0,91	1,02
At1g71730	1379	0,892	6	unknown protein	not assigned.unknown	0,97	1,10
At3g08640	1690	0,860	6	unknown protein	not assigned.unknown	1,31	0,94
At2g26110	1857	0,922	6	unknown protein	not assigned.unknown	1,20	1,04
At4g36410	935	0,868	6	E2, ubiquitin-conjugating enzyme 17 (UBC17)	protein.degradation	0,76	0,90
At1g23410	1975	0,850	6	ubiquitin extension protein, putative	protein.degradation	0,35	0,48
At1g49580	856	0,886	6	CDPK-related protein kinase, putative	protein.postranslational modification	0,57	1,09
At1g03740	735	0,883	6	putative protein kinase	protein.postranslational modification	1,13	0,86
At1g05000	1703	0,906	6	unkown protein	protein.postranslational modification	1,24	1,47
At1g56110	1082	0,899	6	SAR DNA binding protein, putative	RNA.regulation of transcription	0,96	0,54
At3g51880*	738	0,913	6	high mobility group protein 2-like	RNA.regulation of transcription	1,21	1,19
At4g00870*	1432	0,858	6	similar to the myc family of helix-loop-helix transcription factors	RNA.regulation of transcription	2,00	2,50
At3g01460	1272	0,867	6	unknown protein	RNA.regulation of transcription	1,37	1,23
At2g40230	1815	0,871	6	putative anthranilate N-hydroxycinnamoyl/benzoyltransferase	secondary metabolism.phenylpropanoids	0,69	0,89
At4g14640	865	0,871	6	calmodulin	signalling.calcium	0,48	0,79
At5g60900	947	0,892	6	S-receptor kinase homolog 2 precursor	signalling.receptor kinases	1,76	0,53
At5g49770	775	0,891	6	receptor protein kinase-like	signalling.receptor kinases	1,41	0,14
At3g55740	1403	0,859	6	proline transporter 2	transport.amino acids and peptides	2,23	1,25
At5g13780	1012	0,856	7	putative protein	development.unspecified	1,04	0,93
At2g43020	925	0,870	7	putative amine oxidase	misc.oxidases - copper, flavone etc.	1,20	0,95
At3g49960	794	0,857	7	peroxidase ATP21a	misc.peroxidases	0,71	0,73
At5g60120 class 7 (14 NEIGHBOURS)							
At1g53050	403	0,859	6	cell division-related protein, putative	cell.division	0,97	1,32
At1g73920	412	0,866	6	putative lipase	lipid metabolism.lipid degradation.lipases	1,19	1,03
At3g23600	1154	0,853	6	unknown protein	misc.misc2	1,70	1,05
At4g12280	122	0,864	6	copper amine oxidase like protein (fragment2)	misc.oxidases - copper, flavone etc.	3,13	0,94

At5g19050	210	0,922	6	putative protein	not assigned.unknown	1,17	1,13
At2g36810	91	0,887	6	unknown protein	not assigned.unknown	1,24	1,15
At1g22750	155	0,893	6	unknown protein	not assigned.unknown	1,09	0,97
At1g76850	234	0,890	6	unknown protein	not assigned.unknown	1,13	1,17
At1g58250	309	0,906	6	unknown protein	not assigned.unknown	1,07	1,07
At5g48930	304	0,873	6	anthranilate N-benzoyltransferase	secondary metabolism.phenylpropanoids	1,82	1,14
At4g38350	175	0,956	7	putative protein	not assigned.no ontology	1,16	1,09
At5g03900	226	0,885	7	putative protein	not assigned.unknown	1,22	1,03
At5g63940	45	0,916	7	putative protein	protein.postranslational modification	1,78	1,18
At5g58270	212	0,855	7	ABC transporter-like protein	transport.ABC transp and multidrug resistance systems	1,19	1,01

APPENDIX E Commonly used nucleotides

Primers used for PCR screening of T-DNA knock-out lines			
Name	**Sequence (5' – 3')**	**Name**	**Sequence (5' – 3')**
SALK_3_F	TCCAACCAATAGCCTCCCGAACTT	SALK_3_R	TTTGGGTGGGGGAAGGAAGAAGGA
SALK_5_F	CGACCTACGAAGGCGTTCATA	SALK_5_R	TGGAGTTGCCTGAGAAGAGGA
SALK_8_F	ACGAGCGGTTGTACCAAACGT	SALK_8_R	AAACCGCGTCTTCGAGCAA
SALK_9_F	CAGCCGAAGAAGAGCAAACAATCC	SALK_9_R	GCGTCGCAATAGCTGACCATTTA
SALK_13_F	ATTGGTCGGCCGTAAGTAATGA	SALK_13_R	TCCACGGTGGCATCAATGT
SALK_17_F	TTATGCGGCAACACCTCAACCACC	SALK_17_R	TATAAGGGAGAAGACCCTGGCGGA
LBb1	GCGTGGACCGCTTGCTGCAACT		
Q-RT-PCR primers for non-TF genes			
Name	**Sequence (5' – 3')**	**Name**	**Sequence (5' – 3')**
Sultr1;1_F	TTCTGTCATGCACTCCGTATTCAA	Sultr1;1_R	TGCCAATTCCACCCATGC
Sultr1;2_F	TCACCCTGTGGACGGAAGTC	Sultr1;2_R	GTTTCATCGGAACATGTCCACC
Sultr1;3_F	GGCGATTACCTTCTCAAGGGCTTC	Sultr1;3_R	TCCTATCGCTACAGCTTCCGTCAA
Sultr2;1_F	ATTGTTGCTCTAACCGAGGCGATT	Sultr2;1_R	TGTACCCTTTTATTCCGGCGAACG
Sultr2;2_F	CGGTTAGTCATCGCTAGTCCCAGA	Sultr2;2_R	TCTCGTCCAATTTTGCTCGCTTCA
Sultr3;1_F	TCGTCGTAGCGGTGGCGATATCTA	Sultr3;1_R	ACCGCAGTTTTTGGCCTCGACA
Sultr3;2_F	TCACTACCGGGCCATTTTCACGTT	Sultr3;2_R	TGCAACCGCCATCACCACGTTT
Sultr3;3_F	AATGTCAGCCGTGAGCGGTGTA	Sultr3;3_R	ACACAAGCTCGATGTCCTTCTTGG
Sultr3;4_F	TCTTCAGCTTGTGCTGGTGAATCC	Sultr3;4_R	TCCACTCAGACCCAATGCCTCAAT
Sultr3;5_F	CAACGGGGCCATTTTCAAAGACAG	Sultr3;5_R	AGAAGCACAAGCATCATGCAAACG
Sultr4;1_F	GAGATCGGTGTCCTTGTTGGTGTT	Sultr4;1_R	CCCAAGACAGCAATGTGAGGGTT
Sultr4;2_F	ACCACAGTGTGCTTTAGCAGCAAT	Sultr4;2_R	TCTCTTGTCCACACGCCACAGA
Sultr5;1_F	AAGCCGCTCCTCGTGATGTCTAAG	Sultr5;1_R	AGCTGCCCGCCATTTCGTCATAAG
Sultr5;2_F	AAATGCTGCCATAGGCTTTGTTGC	Sultr5;2_R	CCCGTAGTTCCGCATCCACAAAAC
ATPS1_F	TGTTCATCTCCGGCACTAAGATGC	ATPS1_R	TCCACCAGAACTTTCCATCCACCT
ATPS2_F	TGGTTCTCTCCGAAGTGTGGAGAT	ATPS2_R	TCCTGGAGAAGTAGTTCCCCAAGT
ATPS3_F	GGCACTAAGATGAGAGCATTGGCA	ATPS3_R	ATCCACAAGGACTTTCCAGCCTCC
ATPS4_F	AAGATGCGTGGGTTGGCGAA	ATPS4_R	CAACCAGAGGGACACATGAAACCA

APSK1_F	GAGCCACCATTGAACTGCGAGAT	APSK1_R	ATCCGACGACCTTTTCCGCCAT
APSK2_F	GCTGATTTTCCCGCCCTTTCAGAA	APSK2_R	GCATATCGAACTCTCGTGCCACAC
APR1_F	TCATTGGAGCCAAAAGTTTCGCAA	APR1_R	TCAGAGACACAGGAGCAACATGAA
APR2_F	TGATCGAACCCATTTGTCTCAGAG	APR2_R	TCAGGAGCAATTAGAGTTGAAGCA
APR3_F	GGAAGAGATCCTCCGTGAAAGC	APR3_R	CTGTAACCTCAGAAGCAACAATGGA
SAT_F	TGTGGAAGCAAGGAAGAAAACT	SAT_R	GGGATGAATATCGATTCCAAAA
SAT 1_F	GATGTCTGGATCAAGATGCTTGAAG	SAT 1_R	GGTTCTTGTTTAACATCGGATTTGG
SAT A_F	TGTTTGGGCCAAAATCCGAGAAGA	SAT A_R	CGCTGAGAAACAATCGAAGCGTGA
SAT2 106_F	AGCCGAATAAGCGAGGTTTT	SAT2 106_R	TGACCACTCCAGTTCCATGA
SAT52_F	ACAAGCTATGGACACAATCACG	SAT52_R	AACAGCGAATACATCGGAGATT
1OASTLA1_F	CACGAGCGATTTTCTCCATT	1OASTLA1_R	CAATTCTCGAGGCCATGATT
2OASTLB_F	GCTGCTTTCGATGTTTCCTC	2OASTLB_R	ACACCAGCTTCTGGCTTGAT
3OASTLC_F	GAGGCAGAGTCGGAGTTTTG	3OASTLC_R	TGTTGAGTCCATCAGGTCCA
4OASTL26_F	CTACCGGAACGAAATCGAAA	4OASTL26_R	TGTTGCTCTTCAGCCACAGT
5OASTLD1_F	TTGTCGACAGTCTTCTTCTTCTTC	5OASTLD1_R	GGTCCTCCTCCATGTCAAAA
6OASTL_F	ATGGCTTCAGTGGCTCCTAA	6OASTL_R	CAGCAACATGACCAACACAA
7OASTLC1_F	CCACACCGTGAGGAAACTCT	7OASTLC1_R	GCGTTGGTAGAGGGGAAGTC
8OASTLD2_F	TGTTAGCAAACTTTGACCATTGA	8OASTLD2_R	TGGATTTATGAAGTCCAATAACCAT
10OASTLn_F	TCCGGAGGAAATCTGTTCTG	10OASTLn_R	TGCCGCAATGAAGATGATAA
vikin-like1_F	TTCCGAAGTGGACGAGCTACGA	vikin-like1_R	CCGCCACAACTGCAACATCTCTTT
IFR_F	CCAAGAGTCTCCGATTCCCAT	IFR_R	CCATTCACAAACACCGCGT
sugar_tr_F	TTTGGAGCCCAAGCGGGACTTT	sugar_tr_R	CGGCACCAAGCACCATGTGAATAC
ChaC-like_F	ACATCCTGCTAGGACTTGTACGCT	ChaC-like_R	AGCAGCACCCCACTAAATAGCTCC
SHMTs_F	AGGTGTAAGAATAGGGACACCCGC	SHMTs_R	GCCGCCTTTATCAGAAAATCCGC
NIT3_F	CTGGCCCTGAAGTGGAAAGATT	NIT3_R	CCCCCATTACCAAGTGCACAT
isochorismate_synth_F	TAGCACGGTTGCAGTGTGAAGG	isochorismate_synth_R	TGGAAGAGTCTCTCAGGCGTGTTC
CSD1_F	TCGACATGCTGGTGATCTAGGA	CSD1_R	TGTGAAGGTGGCAGTTCCATC
OPR1_F	TCTCTTTACCCCTCCAAGACGG	OPR1_R	CTCCATCAAAACCAGCTTCCAT
OPR2_F	TGGCATTGATGAAGCTCGCT	OPR2_R	CTCCATCAAAACCAGCTTCCAT
OPR3_F	TCTCTCATCGAGTGGTTCTGGC	OPR3_R	TTTGGTACTCCGTTCAACGCC
DHAR_F	CGACAAACCCCAATGGTTCTTA	DHAR_R	ATTTGCCATCAAGCTTCACCAC
CYP79B3_F	CCAGCCTTTGCTTACCGCTGAT	CYP79B3_R	GCGCCGCCATTACAAGTTCCTTAA

UBQ10 new_F	GGCCTTGTATAATCCCTGATGAATAAG	UBQ10 new_R	AAAGAGATAACAGGAACGGAAACATAGT
UBQ10 old_F	CACACTCCACTTGGTCTTGCGT	UBQ10 old_R	TGGTCTTTCCGGTGAGAGTCTTCA
EF1a_F	TGAGCACGCTCTTCTTGCTTTCA	EF1a_R	GGTGGTGGCATCCATCTTGTTACA
ACT2_F	TCCCTCAGCACATTCCAGCAGAT	ACT2_R	AACGATTCCTGGACCTGCCTCATC
GAPDH5'_F	TCTCGATCTCAATTTCGCAAAA	GAPDH5'_R	CGAAACCGTTGATTCCGATTC
GAPDH3'_F	TTGGTGACAACAGGTCAAGCA	GAPDH3'_R	AAACTTGTCGCTCAATGCAATC
INTRON_SEQ_F	TTTTTTGCCCCCTTCGAATC	INTRON_SEQ_R	ATCTTCCGCCACCACATTGTAC
At2g28550(63)-F	GCCGAGGGAAGAGCAACAGAAAAG	At2g28550(63)-R	TCTGCCATCCCCAGTTACTCATCA
At5g60120(70)-F	GGCAGCTAATGTTAAGCTCGACCT	At5g60120(70)-R	TGCTTCGGACCATCTCCTAGTGAA
SALK_3_F	TCCAACCAATAGCCTCCCGAACTT	SALK_3_R	TTTGGGTGGGGGAAGGAAGAAGGA
SALK_5_F	CGACCTACGAAGGCGTTCATA	SALK_5_R	TGGAGTTGCCTGAGAAGAGGA
SALK_8_F	ACGAGCGGTTGTACCAAACGT	SALK_8_R	AAACCGCGTCTTCGAGCAA
SALK_9_F	CAGCCGAAGAAGAGCAAACAATCC	SALK_9_R	GCGTCGCAATAGCTGACCATTTA
SALK_13_F	ATTGGTCGGCCGTAAGTAATGA	SALK_13_R	TCCACGGTGGCATCAATGT
SALK_17_F	TTATGCGGCAACACCTCAACCACC	SALK_17_R	TATAAGGGAGAAGACCCTGGCGGA
		Other primers	
Oligo dT	TTT TTT TTT TTT TTT		

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COMMONLY USED ABBREVIATIONS

ACT2	actine 2
AP	apetala
APSK	APS kinase
APR	APS reductase
APS	adenosine 5'-phosphosulfate
ATP	adenosine triphosphate
ATPS	ATP sulfurylase
bHLH	basic helix-loop-helix
bp	base pair
CbL	cystathionine-β-lyase
cDNA	complementary DNA
CgS	cystathionine-γ-synthase
CHES	N-cyclohexyl-2-aminoethanesulfonic acid
CoA	coenzyme A
Col	Columbia
DAS	days after sowing
DAT	days after transfer
DNA	deoxyribonucleic acid
dNTP	deoxyribose nucleotide triphosphate
DTT	dithiotreitol
DW	dry weight
γ-EC	γ-glutamylcysteine
EDTA	ethylenediaminetetraacetic acid
ERF	ethylene responsive factor
EST	expressed sequence tag
FW	fresh weight
gDNA	genomic DNA
GSH	glutathione (reduced form)
GSSG	glutathione (conjugated)
Hcy	homocysteine
HPLC	high performance liquid chromatography
kb	kilo bases
KO	Knock Out
LB	left border
M	molar, molarity
mBBr	3-bromomethyl-5-ethyl-2,6-dimethyl-pyrazolo(1,2-α)pyrazol-1,7-dione
mRNA	messenger RNA
½ MS	half-strength Murashige and Skoog medium
N	Nitrogen
OAS	*O*-acetyl-serine
OASTL	*O*-acetyl-serine(thiol)-lyase, OAS(thiol)-lyase
OPA	ortho-phthaldialdehyde
OPHS	*O*-phosphohomoserine
OX	over-expressor
P	phosphate
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PCR	Polymerase Chain Reaction
PS	photo system

PVPP	polyvinylpolypyrrolidone
qRT-PCR	Quantitative Reverse-Transcription Polymerase Chain Reaction
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature, reverse transcriptase
+S	normal sulfur (condition)
–S	sulfur deficiency
S	sulfur
SAM	S-adenosyl-methionine
SAT	serine acetyltransferase
SD	standard deviation
SMM	S-methyl-methionine
SULTR	sulfate transporter
Taq	*Thermophilus aquaticus*
T-DNA	transfer DNA
TF(s)	Transcription Factor(s)
UBQ	ubiquitin
UTR	untranslated region
UV	ultra violet
V	volt
wt	wild type
Δ	difference

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