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Functional analysis of selected DOF transcription factors in the model plant *Arabidopsis thaliana*.

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und unter Verwendung keiner anderen als den von mir angegebenen Quellen und Hilfsmitteln verfasst habe.
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Aleksandra Skirycz Potsdam, 08.10.2007

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Abbreviations

ACT - arabidopsis co-expression tool
CDF1 – cycling DOF factor 1
CDK – cyclin dependent kinase
ChIP – chromatin immunoprecipitation
cry1 – cryptochrome 1
CYC - cyclin
DEX – dexamethasone
DOF – DNA binding with one zinc finger
EMSA – electrophoretic-mobility shift assay
ESR2 – enhanced shoot regeneration 2
GC-MS – gas chromatography coupled to mass spectrometry
GFP – green fluorescent protein
GR – glucocorticoid domain
GS - glucosinolates
GST6 – glutathione S-transferase 6
HPLC – high pressure liquid chromatography
IAA – indole-3-acetic acid
IAOx - indole-3-acetaldoxime
IGS – indole glucosinolates
IOE – inducible over-expression
KRP – Kip related protein
LC-MS – liquid chromatography coupled to mass spectrometry
MeJA - methyl jasmonate
NAA - 1-naphthaleneacetic acid
OBF4 – *ocs* element binding factor
OBP – OBF4 binding protein
OE – over-expression
PBF – prolamin-box binding factor
PCA - principle component analysis
phyB – phytochrome B
RAM – root apical meristem
SA - salicylic acid
SAGE - serial analysis of gene expression
SELEX - systematic evolution of ligands by exponential enrichment
SAM – shoot apical meristem
Q-PCR - quantitative polymerase chain reaction
Q-RTPCR – quantitative real time polymerase chain reaction
TF – transcription factor

1 Summary

Transcription factors (TFs) are global regulators of gene expression playing essential roles in almost all biological processes, and are therefore of great scientific and biotechnological interest. This PhD project focused on functional characterisation of three DNA-binding-with-one-zinc-finger (DOF) TFs from the genetic model plant *Arabidopsis thaliana*, namely OBP1, OBP2 and AtDOF4;2. These genes were selected due to severe growth phenotypes conferred upon their constitutive over-expression.

To identify biological processes regulated by OBP1, OBP2 and AtDOF4;2 in detail molecular and physiological characterization of transgenic plants with modified levels of *OBP1*, *OBP2* and *AtDOF4;2* expression (constitutive and inducible over-expression, RNAi) was performed using both targeted and profiling technologies. Additionally expression patterns of studied TFs and their target genes were analyzed using promoter-GUS lines and publicly available microarray data. Finally selected target genes were confirmed by chromatin immuno-precipitation and electrophoretic-mobility shift assays. This combinatorial approach revealed distinct biological functions of OBP1, OBP2 and AtDOF4;2.

Specifically OBP2 controls indole glucosinolate / auxin homeostasis by directly regulating the enzyme at the branch of these pathways; CYP83B1 (Skirycz *et al.,* 2006). Glucosinolates are secondary compounds important for defence against herbivores and pathogens in the plants order *Caparales* (e.g. Arabidopsis, canola and broccoli) whilst auxin is an essential plant hormone. Hence OBP2 is important for both response to biotic stress and plant growth.

Similarly to OBP2 also AtDOF4;2 is involved in the regulation of plant secondary metabolism and affects production of various phenylpropanoid compounds in a tissue and environmental specific manner. It was found that under certain stress conditions AtDOF4;2 negatively regulates flavonoid biosynthetic genes whilst in certain tissues it activates hydroxycinnamic acid production. It was hypothesized that this dual function is most likely related to specific interactions with other proteins; perhaps other TFs (Skirycz *et al.,* 2007).

Finally OBP1 regulates both cell proliferation and cell expansion. It was shown that OBP1 controls cell cycle activity at the G1 control point by directly targeting the expression of core cell cycle genes (*CYCD3;3* and *KRP7*), other TFs and components of the replication machinery. Evidence for OBP1 mediated activation of cell cycle during embryogenesis and germination was presented. Additionally and independently on its effects on cell proliferation OBP1 negatively affects cell expansion *via* reduced expression of cell wall loosening enzymes.

Summing up this work provides an important input into our knowledge on DOF TFs function. Future work will concentrate on establishing exact regulatory networks of OBP1, OBP2 and AtDOF4;2 and their possible biotechnological applications.

2. General introduction

2.1 Transcription factors

2.1.1 General characterization

Transcription is the synthesis of RNA from a DNA template catalyzed by the enzyme RNA polymerase. It starts with transcription initiation when RNA polymerase binds to the promoter region at the start of the gene and is followed by elongation and termination. Transcription in eukaryotes is regulated at initiation and consequently this process involves a large number of diverse proteins which facilitate the various expression patterns that are required for biological complexity. Briefly these proteins can be classified into RNA polymerases and general factors that form the basal transcription apparatus; sequence specific DNA-binding transcription factors (TFs); large multi-subunit co-activators and co-factors; and chromatin related proteins such as histone acetyltransferases (Figure 1).

The basal apparatus is required for the initiation of RNA synthesis at all promoters. In contrast, TFs recognize specific DNA sequence elements and are responsible for activating or repressing the expression of specific genes and are therefore responsible for controlling temporal and spatial patterns of transcription. TFs exert their function through direct or indirect (through co-activators) interactions with the basal apparatus that lead to changes in the properties of RNA polymerase (e.g. enzyme efficiency). The domain responsible for this interaction is called the activation domain and in the majority of cases it is separate from the DNA binding domain.

Transcription factor binding sites, called *cis*-regulatory-elements, are usually short (5 – 10bp) and are in most cases shared by a family of TFs. This brings another characteristic of eukaryotic TFs - the combinatorial mechanism of their action. Often it is not a single but rather a combination of several TFs that regulate gene expression. This is an economical and highly dynamic way of selectively regulating expression of tens of thousands of genes with a limited repertoire of TFs and TF binding sites. Multiple stimuli can converge through different *cis*-acting elements on a promoter to coordinately regulate the expression of the corresponding gene.

It is estimated that approximately 5% of eukaryotic genes encode TFs (Riechmann and Ratcliffe, 2000; Riechmann *et al.,* 2000). Based on their DNA binding domain TFs are classified into different families, some of which are found in all eukaryotes (e.g. MYB or bHLH) whilst others are kingdom specific. To date, more than 100 different DNA binding domains have been found (Kummerfeld and Teichmann, 2006). These domains are used to computationally predict TFs in a genome of interest. However, not all DNA binding domains have yet been uncovered.

For example, both yeast and *C. elegans* proteins that bind DNA but that do not possess a known DNA binding domain have recently been retrieved (Hall *et al.* 2004; Deplancke *et al.,* 2006).

Information on TFs, *cis*-elements and transcriptional networks are stored in various databases updated *via* literature searches and refinements of genome annotations. In plants examples include *cis*-element databases such as PLACE (Higo *et al.,* 1999) and PlantCARE (Rombauts *et al.,* 1999; Lescot *et al.,* 2002), transcription factor databases such as PlnTFDB (Riaño-Pachón *et al.,* 2007) and others that contain both types of information, such as the AGRIS database for *Arabidopsis thaliana* (Davuluri *et al.,* 2003; Palaniswamy *et al.,* 2006).

In addition to TFs, there are other mechanisms regulating transcription in eukaryotes such as DNA methylation, chromatin structure and nuclear organization but as these are not the focus of the current work, and they have been extensively reviewed elsewhere (Matzke and Birchler, 2005; Rando, 2007; Razin *et al*., 2007), they will not be described here.

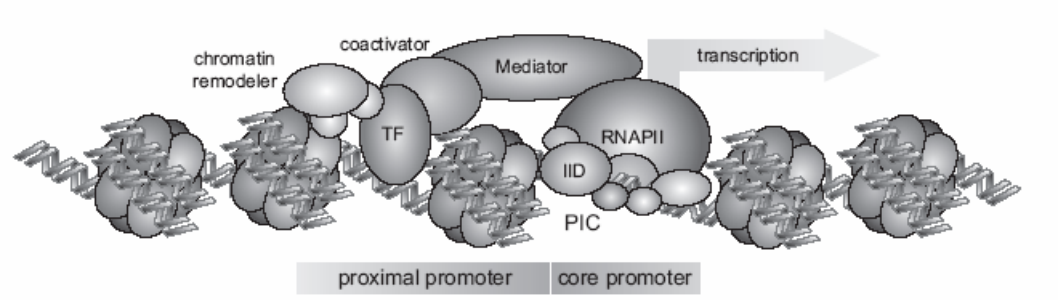

Figure 1. Typical structure of an active eukaryotic promoter of the protein coding genes (reproduced from Heintzman and Ren, 2006). The promoter contains a core promoter region adjacent to the transcription start site and a more distant and longer proximal promoter region. RNA polymerase II (RNAPII) in combination with general transcription factors forms the pre-initiation complex (PIC). Other regulatory proteins such as mediator, coactivators, chromatin remodelers and sequence-specific transcription factors (TF), are involved in regulation of transcription. All of these events occur in the context of chromatin.

2.1.2 Transcription factors in plants

These general characteristics of eukaryotic TFs also hold for plants, however, there is data indicating the proportion of the genome encoding for TFs is slightly higher in plants than in animals. In the model plants Arabidopsis and rice TFs account for approximately 6% and 4.5% of all estimated genes (Riaño-Pachón *et al.,* 2007), respectively, whilst in *Drosophila melanogaster*, *Caenorhabditis elegans*, and yeast the proportions are 4.6%, 3.5% and 3.5%, respectively (Riechmann *et al.,* 2000). The higher number of TFs has often been related to complexity of plant secondary metabolism as well as to the complex environmental interactions necessitated by their sessile lifestyle and occurred due to the extensive genome duplications of

plants (Riechmann, 2002). However, the exact numbers of TFs in Arabidopsis and rice are still not known due to continuous refinement of genome annotations as more experimental data become available. For example the current version of the Arabidopsis transcription factor database, AtTFDB, contains 50 families and 1,690 TFs (Palaniswamy *et al.,* 2006), which is 16 families and 315 TFs more than the previous version (Davuluri *et al.,* 2003). As previously mentioned, whilst plants share certain families of TFs with other organisms (e.g. MYB, bZIP or E2F), many other families are plant specific (e.g. DOF) (Riechmann, 2002). A further difference between transcriptional regulation in plants and animals is the shorter promoters of plants which often span less than one or two kilo bases upstream of the transcription start site (Riechmann, 2002). This likely reflects the higher density of plant genomes. In common with other organisms, there are also occasional examples of promoter sequences that are not directly up-stream of the transcription start site, for example in the intron region of the Arabidopsis gene *AGAMOUS* (Sieburth and Meyerowitz, 1997).

2.2 DOF transcription factors

2.2.1 General characteristics

DNA binding with one zinc finger (DOF) transcription factors are specific for the plant kingdom. They share a highly conserved N-terminal DOF region containing a single Cys2/Cys2 zinc finger motif which acts as a DNA-binding domain (Yanagisawa, 1995 and 1996; de Paolis *et al.,* 1996, Chen *et al.,* 1996). The activation domain is separate from the DOF domain and is found at the C-termini (Yanagisawa, 2001). The conserved DOF domain also reflects a conserved DNA binding site. All DOF proteins analyzed so far, except for one from pumpkin (Kisu *et al*. 1998), recognized an AAAG motif as the essential sequence element in *in vitro* DNA-binding assays (Yanagisawa and Izui, 1993). The specific interaction of DOF domain proteins with the AAAG motif has also been verified *in vivo* (Baumann et *al.,* 1999). In addition, further experiments showed positive effects for the DOF–DNA interaction for tandem repeated DOF elements but limited effects of the DNA sequence bordering the AAAG motif (Yanagisawa and Schmidt, 1999). Considering such a short binding site common for all DOF TFs, combinatorial interactions with other proteins on the promoter sequence together with the position of the core AAAG motif on the nucleosome surface are probably a key for their specificity (Cavalar *et al.,* 2003). DOFs interactions with other DOF TFs (Yanagisawa, 1997), members of other TF families (e.g. bZIP and MYB TFs) and with non-histone nuclear proteins called high-mobility group (HMG) proteins (reviewed by Yanagisawa, 2002 and 2004) were demonstrated. The first

protein–protein interaction was found between the Arabidopsis DOF TF OBP1 and two bZIP proteins associated with stress responses (OBF4 and 5, Zhang *et al*., 1995) and will be described in more detail in the next section. Another example of DOF-bZIP interaction, shown to be important for endosperm-specific gene expression in maize, is between the DOF TF PBF and the bZIP TF OPAQUE2 (Vicente-Carbajosa *et al.,* 1997). Subsequently, the barley ortholog of PBF and other barley DOF TFs (SAD, HvDOF19 and HvDOF17) were also shown to interact with the MYB TF GAMYB on the promoters of endosperm specific genes (Figure 2) (Mena *et al.,* 2002; Isabel-LaMoneda *et al.,* 2003; Diaz *et al.,* 2005; Moreno-Risueno *et al.,* 2007). In another example from maize, the interaction between maize DOF1-2 and HMG proteins facilitates DOF binding to DNA and is dependent on HMG's phosphorylation status (Cavalar *et al.,* 2003).

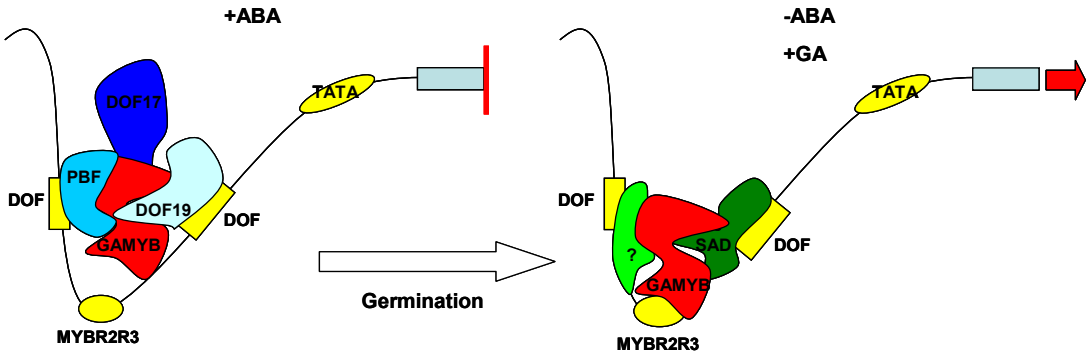

Figure 2. Proposed model of transcriptional regulation of hydrolase genes in barley aleurone layers mediated by DOF TFs and GAMYB (reproduced from Moreno-Risueno *et al.,* 2007). In the early phase of germination when levels of abscisic acid (ABA) are high, negative regulation is exerted by HvDOF17, HvDOF19 and HvPBF. Later during germination the concentration of gibberellins (GA) increases that leads to activation of positive regulators (among them the DOF TF SAD). Transcription factors are indicated as colourful shapes (HvDOF17, HvDOF19, BPBF, GAMYB and SAD) and DNA target sequences as yellow boxes on the DNA strand.

The protein sequence outside the DOF domain is divergent and is related to the functional diversity that arose during evolution. Phylogenetic analysis suggests the origin of DOF TFs likely pre-dates the divergence of the green algae and the ancestors of the terrestrial plants (Moreno-Risueno *et al.,* 2007). Duplications of an ancestral DOF TF gene followed by subsequent neo-or sub-functionalization and pseudogenization account for the expansion of the DOF family (Yang *et al.,* 2006). The number of DOF genes tends to increase with organism complexity going from one in the green alga *Chlamydomonas reinhardtii* to 36 and 30 in the angiosperms *Arabidopsis thalina* and *Oryza sativa*, respectively. A phylogenetic un-rooted tree constructed by Shigyo *et*

al. (2007) based on the alignment of the DOF domain sequences from Chlamydomonas, the moss *Physcomitrella patens* and angiosperms (Arabidopsis and rice) identified two groups of DOF domains referred to as group A and B. DOF domains that could not be classified into these two groups were assigned to a larger, less homologous group C. Group A contained six moss DOF domains, some of the angiosperm domains and the single Chlamydomonas DOF domain. Group B comprised exclusively moss DOF domains, whereas the majority of the DOF domains from the angiosperms and two domains from moss were assigned to group C. Based on the evolutionary events suggested by this phylogenetic tree, the authors speculated that the group of A-type might be associated with the biological processes common among all green organisms, B-type with those specific to moss and the C-type DOF domain proteins being linked to the development of organs in vascular plants. In Arabidopsis there are seven A-type DOF domain proteins, and the physiological functions of four of these genes have been described. COG1 was shown to negatively regulate phytochrome signalling whilst three related genes, CDF1–CDF3, were shown to be involved in photoperiodic flowering (Imaizumi *et al.*, 2005). Hence, it was concluded that group A-type DOF domain proteins might be associated with light regulation mechanisms. On the other hand Group C includes several DOF proteins which are involved in seed maturation and germination such as OsDOF3, barley PBF and Arabidopsis DAG1 and DAG2, and the StDof1 protein which is likely to be related to guard cell-specific gene expression (Plesch *et al.,* 2001; Washio *et al*., 2001; Gualberti *et al*., 2002; Papi *et al*., 2002). It was therefore speculated that there may be a correlation between the diversification of the group C-type DOF domain proteins and the development of organs in higher plants. An overview of the putative functions for different characterised DOF proteins is given in Table 1.

Table 1. DOF TFs characterised in different plant species.

DOF	Biological process	Candidate target genes	Protein Partners	Group [a]	References
Arabidopsis thalina					
AtOBP1	salicylic acid response	*GST6*	OBF4, OBF5	C	Chen *et al.,* 1996
AtOBP3	growth[1], light signalling[2]	*ORG2* and *ORG3*	OBF4, OBF5	C	[1]Kang *et al.,* 2003 [2]Ward *et al.,* 2005
AtDAG1	seed germination	?	?	C	Gualberti *et al.,* 2002;
AtDAG2	seed germination	?	?	C	Papi *et al.,* 2002
AtCOG1	light signalling	?	?	A	Park *et al.,* 2003
AtCDF1	flowering	*CONSTANS (CO)*	FKF1	A	Imaizumi *et al.,* 2005
H.vulgaris					
HvPBF	seed germination (repressor)	aleurone hydrolase genes	HvGAMYB	C	Mena *et al.,* 2002; Isabel-LaMoneda *et al.,* 2003; Diaz *et al.,* 2005; Moreno-Risueno *et al.,* 2007
HvSAD	seed germination (activator)			C	
HvDOF19 HvDOF17	seed germination (repressor)			A	

Zea mais					
PBF	seed germination	*22-kDa zein gene*	OPAQUE2	C	Vicente-Carbajosa *et al.,* 1997
DOF1	primary metabolism, light response	*orthophosphate dikinase, PEPC*	?	C	Yanagisawa and Sheen, 1998; Yanagisawa, 2000
DOF2			?	C	
Others					
OsDOF3	seed germination, GA resp.	*CPD3, α-amylase*	OsGAMYB	C	Washio *et al.,* 2001
NtBBF1	auxin response	*rolB*	?	?	Baumann *et al.,* 1999
StDOF1	stomata opening	*KST1*	?	C	Plesch *et al.,* 2001
AOBP	redox regulation	*ascorbate oxidase*	?	?	Kisu *et al.,* 1998

[a] Classification according to Shigyo *et al.* 2007

2.2.2 OBPs

DOF transcription factor OBP1 (AtDOF3;4; At3g50410) was identified in a cDNA expression library screen for protein partners using labelled protein of the bZIP transcription factor OBF4 (Zhang *et al.,* 1995). *In vitro* OBP1 stimulates OBF4 binding to *ocs* elements found in the cauliflower mosaic virus *35S* and plant *glutathione-S-transferase* (*GST6*) promoters (Chen *et al.,* 1996). Promoter analysis identified a conserved DOF *cis*-element in the vicinity of the *ocs* elements to which binding was demonstrated by EMSA experiments (Chen *et al.,* 1996). Subsequently two other DOF transcription factors, named OBP2 and OBP3, were shown to have similar properties (Kang and Singh, 2000). They all bind to OBF4 in GST pull-down assays and enhanced OBF4 binding to *ocs* elements in the *GST6* promoter. In the same study *OBP1-3* gene expression was shown to be auxin and salicylic acid responsive with more pronounced induction in the root compared to the shoot.

OBP1 and OBP2 have both paralogs in Arabidopsis; OBP1 - AtDOF5;8 (At5g66940) and OBP2 - AtDOF2;2 (At2g28810) that share 46% and 54% similarity at the protein level, respectively. Using Plant Transcription Factor Databases website we also found orthologs of OBP1 in poplar, cotton, grape and potato but not in rice (Table 2). In contrast *OBP3*, is a single copy gene, with no orthologous genes in any other of the examined plant species.

Table 2. Orthologs of OBP1 retrieved from Plant Transcription Factors Databases website (http://planttfdb.cbi.pku.edu.cn/).

Species	ID	Score ratio	Coverage	Identity	E_value
Poplar	eugene3.00070983	0.47	0.94	0.53	2e-68
Gossypium hirsutum	PTGh00349.1	0.43	0.99	0.52	2e-61
Solanum tuberosum	PTSt00328.1	0.41	0.99	0.49	7e-59
Vitis vinifera	PTVv00189.1	0.4	0.99	0.47	1e-56

Previously, only OBP3 was characterised in more detail. Constitutive over-expression of *OBP3* resulted in a severe growth phenotype with drastically reduced root and shoot size (Kang and Singh, 2000). Expression analysis of transgenic plants (RNAi, 35S and inducible over-expression lines) and the response to salicylic acid / jasmonate identified three putative direct target genes (unknown protein and two bHLH TFs) (Kang *et al.,* 2003). Interestingly the unknown protein shares similarity with yeast cell cycle protein cdc2 whilst bHLH TFs share similarity with MYC proteins involved in both cell proliferation and apoptosis in animals. Independently of these results OBP3 was picked up in a screen for novel components of light signalling (Ward *et al.,* 2005). OBP3 gain of function mutant, *sob1-D*, suppressed the long hypocotyl phenotype of the phytochrome B (phyB) mis-sense allele, *phyB.* Conversely, *RNAi-OBP3* plants were characterised by a longer hypocotyl when grown in red light, and this phenotype was *phyB* dependent. It was concluded that OBP3 is a positive regulator of phyB mediated inhibition of hypocotyl elongation. Moreover, cotyledons of light grown *RNAi-OBP3* plants were larger and this phenotype was especially pronounced in blue light and required functional cryptochrome 1 (cry-1). This indicated that OBP3 may also be a negative regulator of cry-1 mediated cotyledon expansion.

2.3 Methods for the functional characterization of transcription factors

Although the majority of TFs can be annotated relatively easily due to the presence of their conserved DNA binding domains their functional analysis can be difficult. Transcriptional networks are highly complex both due to the regulatory interactions of multiple TFs on a single promoter and because of the dynamic nature of developmental and environmental responses. Moreover, the only available method allowing identification of TF binding sites on a genome-wide scale - chromatin immuno-precipitation - is technically demanding and the results are not always easy to interpret. Commonly used gain- and loss-of-function approaches often provide good biological insight into TF function but usually do not allow primary and secondary interactions to be distinguished. This is why a combination of methods is usually needed to for TF characterization. Lately experimental approaches, facilitated by the growing amount of publicly available micro-array and sequence data, are commonly assisted by bioinformatics tools such as co-expression analysis and phylogenetic foot-printing. An overview of methods used for the functional characterization of TFs will be presented in the following sections, with an emphasis on their applicability in plants.

2.3.1 Identifying putative binding sites

As transcription factors exert their function by binding to specific DNA sequences, the identification of sequences bound by a TF is a good starting point for identifying putative binding sites. The most commonly used methods are based on SELEX (systematic evolution of ligands by exponential enrichment) which uses a purified TF protein to enrich short nucleotide sequences that bind tightly to it from a large initial pool of random synthetic DNA fragments. After several rounds of selection the nucleotide sequences of the tightly bound DNAs are determined and a consensus DNA recognition sequence is formulated. Binding to such consensus sequences may subsequently be confirmed by electrophoretic-mobility shift assay (EMSA). EMSA relies on a difference in the mobility between free and protein bound DNA fragments during gel electrophoresis. The exact sequence requirements for the bound motif can be further investigated by the mutation of individual nucleotides and using these modified sequences to compete for the binding of the TF protein. Examples of SELEX usage in plants include Arabidopsis AP2 TF AINTEGUMENTA (Nole-Wilson and Krizek, 2000) and tomato zinc-dependent DNA-binding protein ASR1 (abscisic acid stress ripening) (Kalifa *et al.,* 2004).

2.3.2 Identifying transcription factors targets

As mentioned previously, putative DNA binding sites are often very short and in most cases shared by a family of TFs. Finding functional binding sites is therefore a necessary step to confidently ascertain transcriptional networks.

2.3.2.1 Transgenic approaches combined with expression profiling

The combination of transgenic approaches with expression profiling technologies is commonly used to identify potential TF targets, particularly for organisms for which transformation is relatively easy such as plants. Both activation and repression of TF activity is used and will be discussed in more detail below (reviewed by Zhang, 2003). Briefly, inactivation of a gene and its encoded protein is generally believed to be more specific as it occurs in the right developmental and/or environmental context whilst activation is usually ectopic. However, also for inactivation, the response can still be indirect as the system responds and adjusts to the modification. This is most evident for proteins that are functionally redundant, which is common in plants due to genome duplications events. The main concern for TF over-expression is a loss of specificity. When present in large excess, and in tissues where it is not usually expressed, transcription factors may confer new functions. Alternatively, due to the lack of co-factors it may stay inactive when studied outside of its usual developmental and environmental context. In

general transgenic approaches (with one exception mentioned below) do not distinguish primary and secondary targets of a TF and long-term effects of changed gene expression may be highly pleiotropic. However, they provide good evidence on regulatory networks and biological processes in which a given TF may be involved. They are also widely used in studying dynamics of transcriptional networks by introducing acute perturbations.

Activation studies

Typically activation of a transcription factor is achieved by either constitutive or inducible over-expression conferred by a strong, ubiquitous promoter such as the cauliflower mosaic virus 35S promoter used in plants. Alternatively, a tissue specific promoter can be used. In the case of post-translationaly regulated TFs certain modifications may be required for full activation. Good examples are plant *E2F* TFs that need to be over-expressed together with their co-activator proteins DPa or DPb (Vandepoele *et al.,* 2005). Although it often results in pleiotropic phenotypes and thus a mixture of primary and secondary effects, constitutive over-expression was for a long time the method of choice. Moreover, it is not always easy to compare wild type controls and transgenic organism due to developmental differences. These problems can be overcome with inducible activation of a TF. The simplest inducible system, widely used in plants, uses a fusion between the TF of interest and a steroid-binding domain. In the absence of the inducer the fusion protein is localized in the cytoplasm and is therefore inactive (Lloyd *et al.,* 1994). Adding of the steroid induces conformational changes in the steroid-binding domain resulting in the migration of the fusion protein into the nucleus where it can function. One advantage of this system, besides its simplicity, is that direct and indirect effects of TF activity may be separated by using inhibitors of protein synthesis (such as cycloheximide). However, cycloheximide treatment itself causes thousands of expression changes (data from AtGeneExpress Chemical Series by Vinegar and Winter, 2004) and so it is not often used. Therefore, a typical experimental setup involves induction, preferably at the developmental stage that TF is biologically relevant, followed by harvesting and expression analysis at different times following induction. It is also crucial (using appropriate controls) to exclude that the changes are not caused by application of steroid (or protein synthesis inhibitors if used).

Other commonly used inducible systems are not restricted to nuclear proteins and rely on transcriptional regulation. They were reviewed by Padidam (2003) and so will only be discussed briefly. Some of these systems use a chimeric TF that when induced migrates to the nucleus activating the transcription of the gene (TF) of interest, whilst others utilize ‘stimulus’ activated

promoters. The inducers range from hormones (estradiol) and chemicals (ethanol) to stress stimuli such as wounding or heat. Examples are given in Table 3.

Table 3. Examples for transcription factors for which target genes were characterized using constitutive or inducible over-expression.

TF	Biological process	Candidate target genes	References
Constitutive over-expression using strong promoters			
CBF2	Response to cold stress	cold-responsive genes (COS)	Vogel *et al.,* 2005
PAP1	Flavonoid Biosynthesis	*DFR, LDOX, TT19, CHS, CHI*	Toghe *et al.,* 2005
E2Fa/DPa	Cell Cycle	replication machinery: *MCMs, PCNA*	Vandepoele *et al.,* 2005
Inducible over-expression (TF fused to GR domain or estradiol domain)			
CONSTANCE	Flowering	*FT, SOC1*	Samach *et al.,* 2000
ESR2	Shoot regeneration	51 genes e.g. *CUC1, OBP1, CYCD1;1, KRP5, IAA20, CYP78A7*	Ikeda *et al.,* 2006
AGAMOUS	Carpel and stamen development	*GH4, ATH1, STP1/2, CR3, AP3,SEP3*	Gomez-Mena *et al.,* 2005
Inducible over-expression (other systems)			
WUSCHEL	Shoot apical maintenance	*ARR5,6,7,14*	Leibfried *et al.,* 2005

Inactivation studies

Similar to over-expression, inactivation can be achieved in a constitutive or inducible manner and different levels of inactivation can be obtained. Complete inactivation is accomplished by knocking out a gene by either homologous recombination (yeast and mouse) or through random mutation (point mutagenesis or insertional mutagenesis). As the second approach is not target specific collections of mutants are generated and screened for desired phenotype or/and insertion site. Good examples are the plant T-DNA collections SALK and GABI-KAT generated for the scientific community (Alonso *et al.,* 2003; Strizhov *et al.,* 2003). The T-DNA insertion positions in these lines have been determined and are now available for the majority of the Arabidopsis genes. In contrast to complete loss-of-function, different levels of constitutive or inducible transcript repression can be accomplished by RNAi and recently also using microRNA technology. The main advantage of these approaches is that they allow the down-regulation of essential genes and that multiple homologous genes can be targeted (Chuang and Meyerowitz, 2000; Schwab *et al.,* 2006). Unfortunately, the strength of silencing tends to diminish with the subsequent generations, a phenomenon that is still not completely understood.

2.3.2.2 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was first developed for yeast and subsequently transferred to mammalian cells and flies. Initially, ChIP was performed to verify candidate DNA-protein interaction, however, nowadays it is used to identify TF binding sites on a genome-wide scale through the use of microarrays (ChIP-on-chip) and recently with an adapted serial analysis of gene expression (SAGE) protocol (reviewed by Blais and Dynlacht, 2005). ChIP has several features that make it invaluable for deciphering gene regulatory networks. It allows the identification of transcription factor binding sites *in vivo* under various developmental and environmental conditions. However, ChIP also has limitations. On the technical side, it is often limited by the availability of a specific antibody for a given TF, and even when available the endogenous TF protein may be at too low concentration. Another serious limitation of ChIP are false negatives and positives. Negative results are generally not interpretable as the lack of enrichment may be caused by chromatin structure, whilst false positives may result from over-fixation of the samples, or similar to other multi-variate data, from noise. Finally, due to the condition-dependency of TF regulatory interactions, the identified targets are often dependent on the biological conditions studied.

The need for a specific antibody can be overcome by using transgenic approaches. The TF of interest may be fused to an epitope tag for which an antibody is available, and expressed under the control of a chosen promoter. Alternatively, in organisms such as yeast, where homologous recombination can be used for transformation, an epitope tag can be recombined into the gene. Unfortunately, transgenic approaches particularly using strong, constitutive promoters may lead to the problems connected with specificity loss discussed previously (section 2.3.2.1).

There are also two methods that are modifications of ChIP which bypass antibody limitation. The first one, called DamID, involves the expression of a TF linked to a bacterial DNA adenine methyltransferase (Dam), which results in the methylation of DNA adjacent to its targets (van Steensel and Henikoff, 2000; Orian *et al*., 2003; Bianchi-Frias *et al*., 2004). DNA is then sequentially isolated, digested with a restriction enzyme cutting only Dam-methylated DNA, size-fractionated, labeled, and hybridized to a DNA microarray for the identification and quantitation of methlyated loci. The second approach involves coexpression of the *Escherichia coli* biotinylating enzyme BirA, with the DNA-binding protein fused to a biotin acceptor sequence (Viens *et al*., 2004). This generates an *in vivo* biotinylated DNA-bound protein that can be efficiently purified through streptavidin affinity. To date both approaches have been used mainly in flies.

ChIP combined with profiling technologies is a relatively new method and to date there are only two published studies where it was applied to discover TF targets on a whole genome scale in Arabidopsis. In the most recent, Lee *et al.,* (2007) and co-workers used ChIP based mapping of HY5 TF binding sites using a *hy5* loss of function mutant complemented with HY5-HA protein expressed under control of the CaMV 35S promoter, anti-HA antibody and a high-density 60-nucleotide oligomer microarrays covering the entire Arabidopsis genome. These authors found a high number of putative *in vivo* binding sites; approximately 3800, of which two-thirds were located in intergenic regions, where they could function in the regulation of neighbouring genes. To determine the functional relevance of the HY5 binding sites genome-wide expression analysis using wild-type and *hy5* plants exposed to different light conditions was performed. In total, 19% (only about 2-fold more than expected by chance) of the differentially expressed genes had a HY5 binding site in their promoter region, indicating that they are most likely regulated directly by HY5. As expected HY5 binding targets were enriched in early light-responsive genes and transcription factors. It was also demonstrated that *in vivo* association of HY5 with promoter targets is not altered under distinct light qualities or during the light-to-dark transition. Moreover, it was shown that HY5 binding alone is not sufficient for transcriptional activation; most likely other cofactors or modification of HY5 is necessary for its transcriptional regulation function.

2.3.2.3 *In silico approaches*

Recently, co-expression analysis has become a popular approach for investigating functional relationships between genes. It has been demonstrated that genes that participate in the same biological process are often co-expressed and may share a common regulator. Thus it is often assumed that a TF regulon may show co-expression. In some cases, and only when the TF is itself transcriptionally regulated, the TF may also be co-expressed with its target genes. This approach has been used to identify MYB28 and MYB29 as novel regulators of aliphatic glucosinolate biosynthesis (Hirai *et al.,* 2007). There are many co-expression databases available for Arabidopsis including CSB.DB (Steinhauser *et al.,* 2004), the Arabidopsis Co-expression Tool (Manfield *et al.,* 2006) and GeneAngler (Toufighi *et al.,* 2005). However, due to the time taken for transcription and translation TF targets, even transcriptionally regulated TFs, may show a time-shift relative to their targets. Recently, the possibility for such time lags between TFs and their targets was incorporated into a method to identify TF-targets from multiple short time series of the abiotic stress response of Arabidopsis (CERMT) (Redestig *et al.,* unpublished data).

Another, more widely used method is phylogenetic footprinting which is based on the conservation of functional *cis*-regulatory elements in closely related organisms. This approach will become increasingly powerful given the recent advances in DNA sequencing. The basis of this method is that selective pressure causes regulatory elements to evolve at a slower rate than the non-functional surrounding sequence and so the conservation of a given motif can be used to support its functionality. An important factor for the efficiency of finding conserved *cis*-regulatory elements by phylogenetic footprinting is the choice of the species compared. The use of closely related species ("phylogenetic shadowing") may result in relatively low specificity and the use of distantly related species ("phylogenetic footprinting") may result in high specificity, but relatively low sensitivity (Ruvinsky and Ruvkun, 2003). Also the choice of the studied biological process is important as it must be conserved between the species used. Thus, it is most useful with fundamental developmental processes as they are usually the most conserved. In plants, phylogenetic footprinting approaches have to take into account duplication events and so require careful establishment of paralogous and orthologous relationships. The benefits and limitations of phylogenetic shadowing and footprinting in plants were recently discussed for the regulatory regions of *AGAMOUS* and *APETALA3* TFs in different *Brassicaceae* species (Koch *et al*., 2001; Hong *et al*., 2003), and a comparison of promoters belonging to orthologous MADS TF genes from Arabidopsis and poplar (De Bodt *et al*., 2006).

2.3.3 Protein-protein interaction studies

As mentioned before protein-protein interactions are crucial for TF function. Good examples include the above described complexes of DOF and MYB TFs on promoters of endosperm specific genes (reviewed by Yanagisawa, 2004). The methods used for finding protein partners range from the yeast two-hybrid system to immuno-precipitation and will not be discussed here (reviewed by Dziembowski and Séraphin, 2004).

2.3.4 Summary

As mentioned before functional characterization of a transcription factor is not a trivial task and this is reflected by limited information on the majority of TFs. Usually much more is known about the biological function of a given TF rather than on its target genes or protein partners. Good example of TFs with known function but lacking comprehensive molecular characterization are TFs involved in fruit patterning in Arabidopsis, FRUITLESS, SHATTERPROOF and REPLUMLESS (reviewed by Dinneny and Yanofsky, 2005) Refinement of the existing methods

in combination with emerging approaches will most likely contribute to the progress in our understanding of transcriptional regulation.

2.4 Aim of the thesis

One of the main interests of our group is transcriptional regulation. TFs characterized during this PhD project were selected during a larger screen of more than 100 TFs covering several TF families. Promoter GUS and constitutive over-expressing lines were generated and subjected to an initial analysis. Subsequently, TFs with interesting expression patterns and/or leading to severe growth phenotypes were picked for further investigation. Over-expression of both *OBP1* and *OBP2* severely affected growth whilst *35S-AtDOF4;2* plants were bushy and almost sterile. We were interested in the cause of such drastic phenotypes and hoped that this is a good starting point to uncover their biological functions.

The main aim of this work was to perform molecular and functional characterization of the three selected DOF TFs using available *in vivo*, *in vitro* and *in silico* tools.

3. DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in Arabidopsis

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Authors´ contributions

Bernd Mueller-Roeber conceived the idea for the study. The research was designed and planned by Bernd Mueller-Roeber, Isabell Witt and Aleksandra Skirycz. The experimental work was done by Aleksandra Skirycz. The research was supported through collaborations as follows: glucosinolate measurements and herbivore feeding were performed in collaboration with Michael Reichelt, Meike Burow and Jonathan Gershenzon; auxin was determined in collaboration with Jakub Rolcik and Miroslav Strnad; Joachim Kopka performed the metabolite profiling; Maria-Inès Zanor and Isabell Witt prepared the 35S-OBP2 and OBP2-GUS constructs.

3.1 Abstract

- Glucosinolates are a group of secondary metabolites that function as defense substances against herbivores and micro-organisms in the plant order *Capparales*. Indole glucosinolates (IGS), derivatives of tryptophan, may also influence plant growth and development. In *Arabidopsis thaliana*, indole-3-acetaldoxime (IAOx) produced from tryptophan by the activity of two cytochrome P450 enzymes, CYP79B2 and CYP79B3, serves as a precursor for IGS biosynthesis but is also an intermediate in the biosynthetic pathway of indole-3-acetic acid (IAA). Another cytochrome P450 enzyme, CYP83B1 funnels IAOx into IGS. Although there is increasing information about the genes involved in this biochemical pathway, their regulation is not fully understood. OBP2 has recently been identified as a member of the DNA-binding-with-one-finger (DOF) transcription factors, but its function has not been studied in detail so far.
- Here we report that *OBP2* is expressed in the vasculature of all Arabidopsis organs, including leaves, roots, flower stalks, and petals. *OBP2* expression is induced in response to a generalist herbivore, *Spodoptera littoralis* and by treatment with the plant signalling molecule methyl jasmonate, both of which also trigger IGS accumulation. Constitutive and inducible over-expression of *OBP2* activates expression of CYP83B1. In addition, auxin concentration is increased in leaves and seedlings of *OBP2* over-expression lines relative to wild-type, and plant size is diminished due to a reduction in cell size. RNA interference-mediated *OBP2* blockade leads to reduced expression of *CYP83B1*.
- Collectively, these data provide evidence that OBP2 is part of a regulatory network that regulates glucosinolate biosynthesis in Arabidopsis.

3.2 Introduction

Glucosinolates (GS) are a group of secondary metabolites that function as defense substances against herbivores and micro-organisms in the plant order *Capparales* which includes the model plant *Arabidopsis thaliana* and economically important crop species such as oilseed rape (*Brassica napus*), Brassica fodder and vegetables (Brader *et al*., 2001; Fahey *et al*., 2001). Similar to other plant secondary compounds like alkaloids or flavonoids, GS and its degradation products additionally have toxic as well as protective effects (e.g. as flavour compounds and cancer-preventive agents) in higher animals and humans. For that reason there is a strong interest in the ability to regulate and optimise the levels of individual GS tissue-specifically to improve the nutritional value and pest resistance of crops (Mithen, 2000; von Poppel, 2000). Extensive studies on GS metabolism in *Arabidopsis thaliana* have brought

researchers closer to this goal and several of the enzymes responsible for GS production have been identified and characterised (reviewed by Wittstock and Halkier, 2002, Hansen and Halkier, 2005). There is evidence that indole glucosinolates (IGS), derived from tryptophan, not only have an essential function in plant defense, but may also influence plant growth and development. Indole-3-acetaldoxime (IAOx), produced from tryptophan by the activity of the cytochrome P450 enzymes CYP79B2 and CYP79B3, is a precursor for IGS biosynthesis but is also an intermediate in the biosynthetic pathway of the plant hormone indole-3-acetic acid (IAA) (Hull *et al*., 2000; Mikkelsen *et al*., 2000; Zhao *et al*., 2002). CYP83B1, another cytochrome P450 enzyme, channels IAOx towards IGS (Barlier *et al*., 2000). Gain-of- and loss-of-function mutations of any of these enzymes changed both IGS and IAA levels, suggesting overlapping regulation of both pathways (Delarue *et al*., 1998; Bak *et al*., 2001; Zhao *et al*., 2002). Since CYP83B1 acts at a metabolic branch point, it was proposed to be a key enzyme for IAA/IGS homeostasis in plants (Bak *et al*., 2001). However, IAA biosynthesis through the CYP79B2/B3 - IAOx pathway may perhaps not be predominant in Arabidopsis because in *CYP79B2/B3* double null mutants free IAA level was not altered in comparison to the wild-type when plants were grown at 21°C, and was only partially affected at 26°C (Zhao *et al*., 2002). There is also evidence that IGS production is regulated both environmentally and developmentally by different signalling molecules (auxin, methyl jasmonate [MeJA], salicylic acid [SA]) and external stimuli (wounding, pathogen attack). Changes in the IGS level positively correlate with expression of genes crucial for IGS production, suggesting an involvement of transcriptional control in this regulatory network (Brader *et al*., 2001; Mikkelsen *et al*., 2003). However, except for the MYB transcription factor ATR1 which regulates some IGS pathway genes (Bender and Fink, 1998; Celenza *et al*., 2005), transcriptional regulators of this network are unknown.

The Arabidopsis genome encodes ~37 members of the DNA-binding-with-one-finger (DOF) transcription factors that play divergent roles in plant specific processes. The amino acid sequence of DOF proteins is divergent except for the highly conserved N-terminal DOF region that acts as both a DNA-binding and protein-protein interaction domain. This domain binds the conserved DNA *cis* element (A/T)AAAG or its complementary inverse sequence (summarized in Yanagisawa, 2002, and Yanagisawa, 2004).

OBP2, also called AtDof1.1 (At1g07640) was identified as a member of the Arabidopsis DOF transcription factor family (Kang and Singh, 2000). OBP2 physically interacts with OBF4, a BZIP transcription factor, and *in vitro* stimulates its binding to *ocs* elements characteristic for pathogen responsive plant promoters (Zhang *et al*., 1995; Kang and Singh, 2000). The biological function of OBP2 has not been investigated so far.

We are particularly interested in analyzing the role of DOF transcription factors expressed in leaves and have previously provided evidence for their involvement in stomatal guard cell gene expression (Plesch *et al*., 2001). Using reverse transcription / polymerase chain reaction (RT-PCR) we identified *OBP2* among leaf expressed *DOF* genes (data not shown), confirming previous observations (Kang and Singh, 2000). In this study we investigate the expression and function of *OBP2*. It is prominently expressed in the phloem of leaves and other organs. In wild-type plants, expression is stimulated by wounding, MeJA treatment and in response to insect feeding where it precedes increased expression of GS biosynthetic genes and a corresponding accumulation of IGS. Accordingly over-expression of *OBP2* in transgenic plants activates the IGS biosynthetic pathway and RNA interference of *OBP2* leads to reduced expression of *CYP83B1*, a key gene in IGS biosynthesis. Collectively, these data provide evidence that *OBP2* is part of a regulatory network that regulates GS biosynthesis in Arabidopsis.

3.3 Results

3.3.1 Identification of OBP2-regulated biological processes using transcript and metabolite profiling

As part of a wider screen to identify candidate genes regulated by DOF transcription factors we performed Affymetrix expression profiling with the AtGenome array representing ~8.200 genes using constitutive over-expression lines. Specifically, we compared two independent *35S:OBP2* lines to two independent empty-vector control lines and two biologically replicated wild-type samples. Genes were considered as changed if they passed the suggested criteria in the Affymetrix Microarray suite 5 (MAS5) software (significant increase/decrease, appropriate 'present' call and greater than 2-fold change) in all eight comparisons between over-expressing lines and empty-vector control lines or wild type. We identified 89 up-regulated and 43 down-regulated genes in *35S:OBP2* lines. Genes were classified according to their annotated function (see Supplementary Material, Tables S1). Of the up-regulated genes 32 are related to stress responses (Table S2), some are regulated by abiotic stress such as the drought-induced protein D21, dehydrin Xero20, a putative galactinol synthase, heat shock protein 17.6A, heat shock protein 70kD and putative metal ion transporters. However, the majority of these genes have assigned functions in response to biotic stress and defense. These include genes involved in secondary metabolism (FAD-linked oxidoreductase), glycosyl hydrolase family members (1-α-glucosidase), cell wall modification enzymes (thaumatin and xyloglucan endotransglycosylase),

a jasmonate biosynthetic enzyme (12-oxo-phytodienoic acid reductase), proteinases, PR proteins and others such as vegetative storage proteins.

Among these biotic stress genes the most prominent group of up-regulated genes were those encoding proteins of the glucosinolate/myrosinase system. Of the seven up-regulated genes three encoded myrosinase-binding proteins (*MBP*) which possibly regulate myrosinase activity. Also up-regulated were the beta-subunit of tryptophan synthase (*TSB2*), involved in tryptophan biosynthesis and *ATR1* (*AtMYB34*) which regulates the expression of anthranilate synthase, both key enzymes of the tryptophan biosynthetic pathway (Bender and Fink, 1998). Biosynthesis of IGS initiates at the conversion of tryptophan to IAOx, catalyzed by CYP79B2 or CYP79B3 (Hull *et al*., 2000; Mikkelsen *et al*., 2000). Expression of both genes was up-regulated in *35S:OBP2* plants suggesting changes in the IAOx pool.

To complement our transcript analysis we performed metabolite profiling experiments which allowed the detection and quantification of a large number of low-molecular weight compounds from leaf extract, including hydroxyl- and amino acids, sugars, sugar alcohols, organic monophosphates, (poly)amines, and aromatic acids (Roessner *et al*., 2000). We analysed *35S:OBP2* plants using gas chromatography coupled to mass spectrometry (GC/MS) and applied principle component analysis (PCA) to the metabolite data set (Roessner *et al.,* 2001). Metabolite data are given in Supplemental Table S3. The metabolite data for *35S:OBP2* lines are clearly separated from wild-type (Figure S1). The five most important metabolites responsible for this separation were proline, galactinol, an unknown metabolite with high similarity to digalactosylglycerol, an unknown metabolite that may be a product of indole glucosinolate breakdown occurring during sample preparation (beta-D-glucopyranose, 2,3,4,6-tetrakis-O-(trimethylsilyl)-, 1-(trimethylsilyl)-1H-indole-3-acetate) and glutamine. Moreover, transgenic samples clustered according to the level of *OBP2* expression, the stronger lines #1 and #10 formed a cluster separate from the weaker over-expression line #8 (Figure S1). Metabolites responsible for this separation were glycine, galactinol, fructose, raffinose and fumaric acid.

Many of these metabolites are known to accumulate in response to stress (reviewed by Loewus and Pushpalatha, 2000) and furthermore the identification of a putative IGS derivative supported a possible effect on GS metabolism.

3.3.2 Detailed analysis of the GS biosynthetic pathway

Our profiling analyses identified GS metabolism as the most likely biological target of *OBP2* action. Targeted expression analysis on *35S:OBP2* plants using quantitative real-time PCR (Q-

RTPCR) confirmed the increased transcript level of *CYP79B2, -B3* and *ATR1* identified by transcript profiling (Figure 1a) and additionally showed increases for *CYP83B1* and *MAM-1* which were not represented on the array. CYP83B1 is the branching enzyme shifting the endogenous pool of IAOx towards IGS production, whereas MAM-1 catalyses the condensing reactions of the first two methionine elongation cycles in short chained aliphatic GS biosynthesis. Other enzymes from the aliphatic GS pathway, *MAM-L*, responsible for further elongation cycles of methionine (Field *et al*., 2004), *CYP79F1/F2,* that metabolise chain-elongated methionine derivatives into aliphatic oximes and CYP83A1, that funnels these into aliphatic GS (Hansen *et al*., 2001, Naur *et al.*, 2003), were not changed.

To study the effect of these expression changes on GS biosynthesis we measured GS in *35S:OBP2* plants in comparison to control plants. In agreement with the elevated expression of *CYP79B2/B3* and *CYP83B1* total IGS concentration was increased ~2-fold and the concentration of the major IGS indol-3-yl-methyl glucosinolate (I3M) was elevated ~3-fold on a leaf fresh weight basis (Figure 1b; Supplemental Table S4). Concomitant with elevated *MAM-1* expression the aliphatic GS content was increased due to the accumulation of short chained GS (Figure 1c; Supplemental Table S5).

Inducible over-expression and RNA interference (RNAi) were used to modulate *OBP2* expression. We used a dexamethasone (DEX)-inducible system consisting of CaMV 35S-driven expression of *OBP2* fused to a glucocorticoid receptor (GR) domain, which upon DEX-treatment becomes active due to nuclear targeting of the fusion protein (Lloyd *et al*., 1994), whereas the expression level of the chimeric gene itself remains unchanged (as seen in Figure 4d). Two *OBP2-GR* lines were compared to two empty-vector control lines either treated with a control solution or with 50µM DEX. Plant material was harvested 10h and 24h after treatment and used for Q-RTPCR experiments. In comparison to control lines, expression of *CYP83B1* was consistently and significantly increased in all *OBP2-GR* lines 10h and 24h after treatment (Figure 1d). Expression of *CYP79B2, CYP79B3* and *ATR1* were either not changed or increased in both *OBP2-GR* and the empty-vector control lines after DEX-treatment (data not shown). *MAM-1* was not changed in any of the lines tested. Indole GS were increased in a single line which had elevated expression of all three cytochrome P450 enzymes, however, in a separate experiment only *CYP83B1* expression was elevated and correspondingly the GS level was comparable to control situation. In agreement with the absence of changes in *MAM-1* expression the level of aliphatic GS was not changed in any line.

To reduce *OBP2* transcript level we transformed *Arabidopsis* plants with an *RNAi:OBP2* construct (see Experimental procedures). Several independent transformants with reduced

OBP2 transcript level were identified using Q-RTPCR following BASTA selection (Figure 1e). *CYP83B1* was significantly reduced in two lines whilst *CYP79B2* and *B3* were significantly reduced in just one line and *ATR*1 and *MAM-1* were not changed. Also the GS content of *RNAi:OBP2* plants was not significantly different from wild-type (data not shown), possibly due to threshold levels in control of the GS pathway

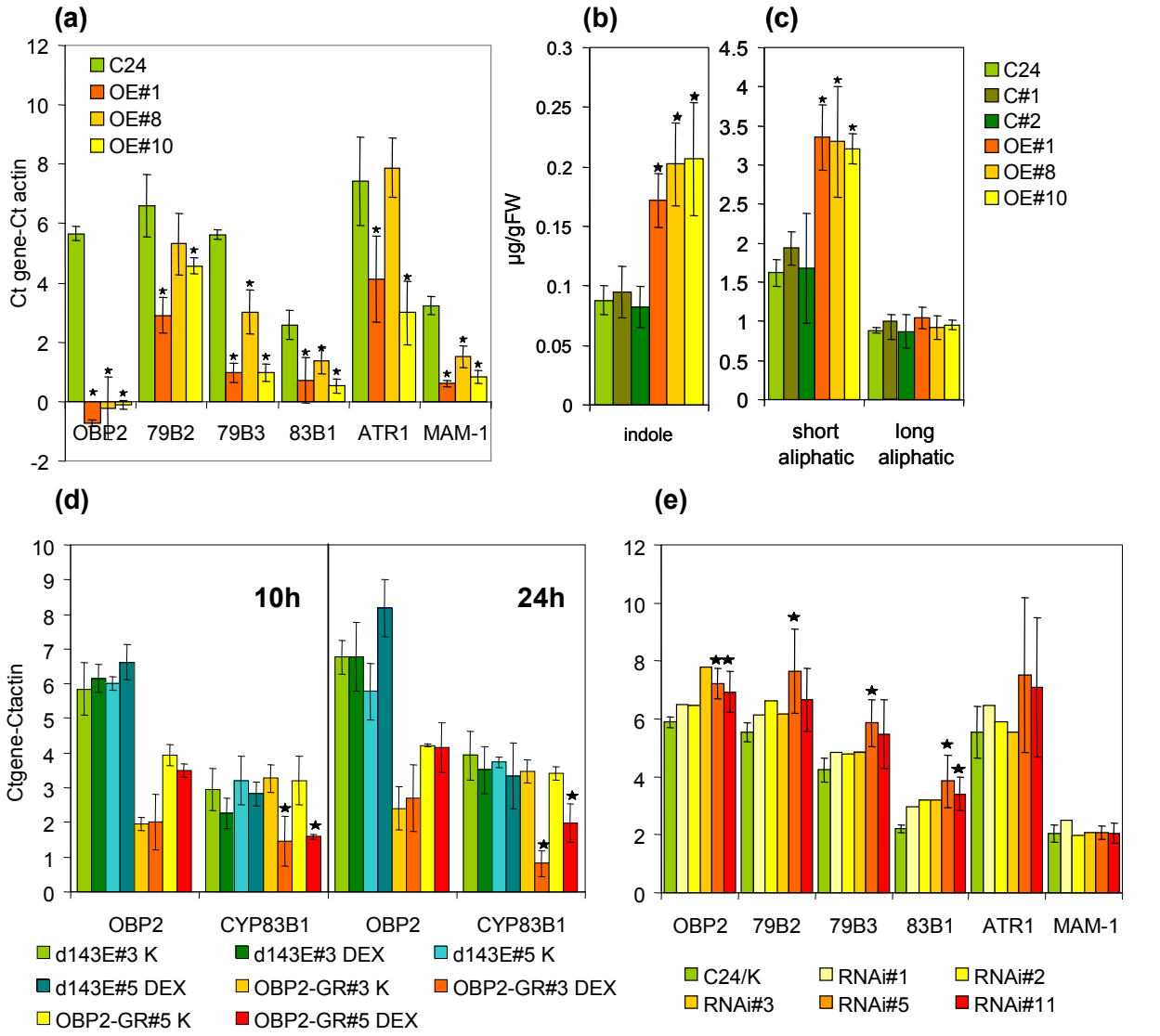

Figure 1. Expression levels of genes involved in GS biosynthesis or its regulation and GS content in transgenic plants with altered OBP2 expression. Transcript level was measured using Q-RTPCR in: (a) *35S:OBP2* (OE) lines #1, #8, and #10; (d) DEX-inducible *OBP2-GR* lines #3 and #5, and empty-vector d143 control lines #3 and #5. K, control treatment with 0.5% ethanol. (e) *RNAi:OBP2* lines #1, #2, #3, #5, and #11. (b, c) GS were determined using HPLC in *35S:OBP2* (OE) plants. Data are mean ± SD (n=3); no

SD is indicated if n=2. *35S:OBP2* and *RNAi:OBP2* expression samples were from three independent experiments pooled from three plants each; all other samples were replicated with individual plants from one experiment. All samples were leaf samples taken from plants prior to bolting. 79B2, *CYP79B2*; 79B3, *CYP79B3*; 83B1, *CYP83B1*. Asterisks (*) indicate values that are significantly different ($p < 0.05$) in comparison to respective controls.

3.3.3 Biological function of *OBP2*

Changes of *OBP2* expression in response to treatments known to stimulate IGS biosynthesis such as MeJA application, wounding and herbivore feeding were measured to investigate their biological significance (Brader *et al*., 2001; Mikkelsen *et al*., 2003; Reymond *et al*., 2004). Northern blot analysis indicated that *OBP2* transcript level increased within four to six hours upon external MeJA application (Figure 2a). Similarly, mechanical wounding, which stimulates jasmonate biosynthesis, enhanced *OBP2* transcript level (Figure 2a). These changes were confirmed by Q-RTPCR experiments indicating a 3-fold induction of *OBP2* transcript level after MeJA treatment (Figure 2c) and a 2-fold induction following wounding (Figure 2d). We also investigated these changes using activity measurements for *promOBP2:GUS* lines (Figure 2b) which indicated that the enhanced *OBP2* expression was at least partly due to transcriptional activation mediated by its promoter. The cellular expression pattern of *OBP2* activity did not change (not shown). In addition we characterized expression of *OBP2* and genes involved in GS metabolism and regulation in leaves damaged by feeding of the generalist herbivore *Spodoptera littoralis*. Samples were taken 0.5, 1, 2, 4, 6, 8, 10 and 24h after the feeding started and 48h after insect removal from the plants. *OBP2* expression increased after 6h, followed by an increase in *ATR1*, *CYP79B2/B3* and *CYP83B1 expression*. These increases were transient with transcripts returning to control levels 24h after the feeding started. There were no changes in transcript level of *MAM-1* (Figure 2e). In agreement with these transcript changes we observed an increase in IGS content from 24h to 72h (Figure 2f) but no changes in the concentration of aliphatic GS (data not shown).

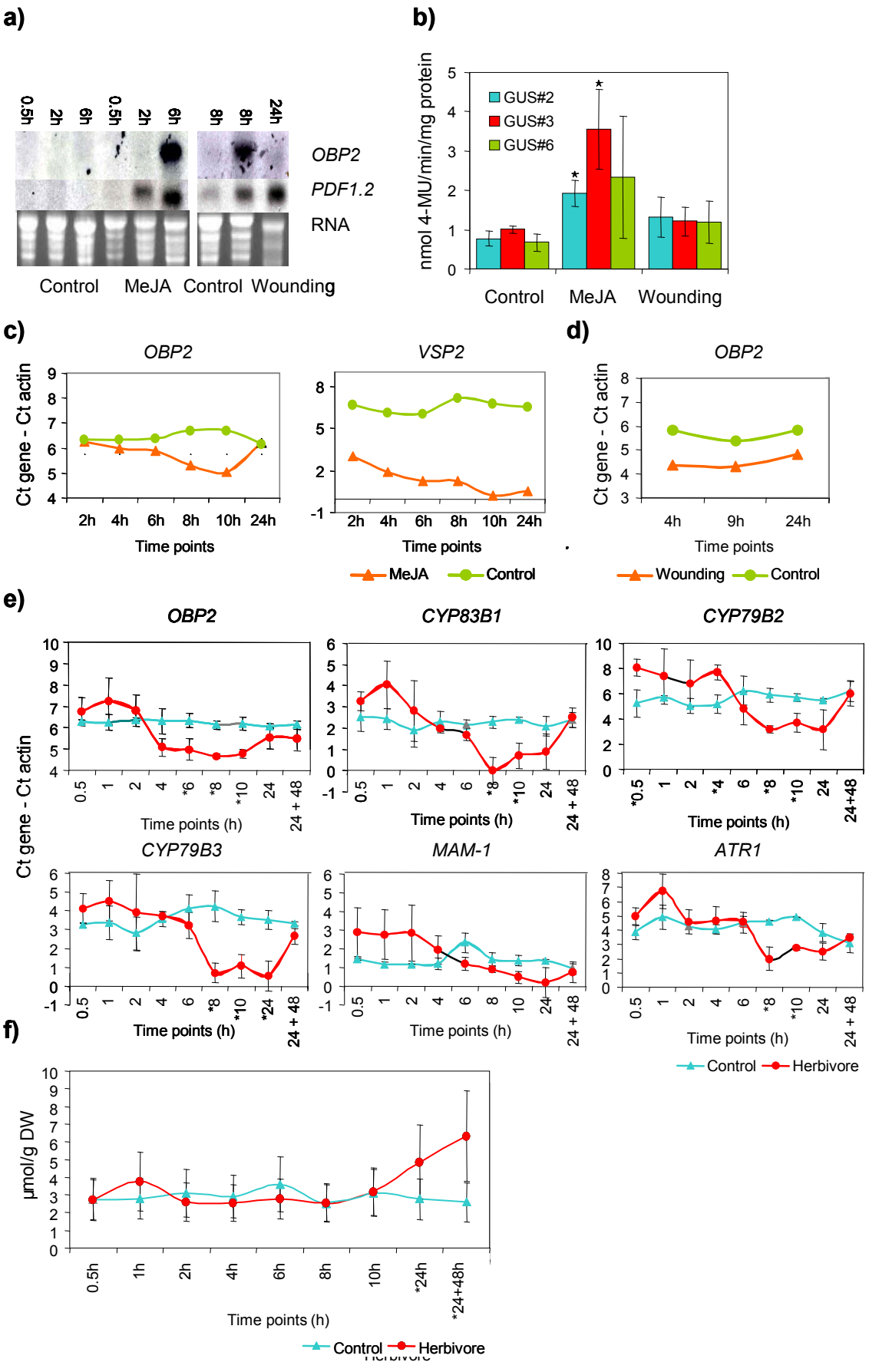
a)
0.5h 2h 6h 0.5h 2h 6h 8h 8h 24h
OBP2
PDF1.2
RNA
Control MeJA Control Wounding
b)
nmol 4-MU/min/mg protein
GUS#2
GUS#3
GUS#6
Control MeJA Wounding
c)
OBP2
VSP2
Ct gene - Ct actin
Time points
MeJA Control
d)
OBP2
Wounding Control
e)
OBP2
CYP83B1
CYP79B2
CYP79B3
MAM-1
ATR1
Time points (h)
Control Herbivore
f)
µmol/g DW
Time points (h)
Control Herbivore

Figure 2. *OBP2* expression is induced by feeding with *Spodoptera littoralis,* MeJA treatment, and wounding.(a) Northern blot analysis of *OBP2* transcript level in Arabidopsis wild-type plants upon MeJA treatment and wounding. RNA was isolated from plants sprayed with 500µM MeJA or 0.006% ethanol solution (control). After spraying, leaf samples were taken at the indicated time points. RNA was isolated from untreated leaves (control) or mechanically wounded leaves of soil-grown plants. The blots were also hybridised to radio-labelled plant defensin 1.2 (*PDF1.2*) cDNA as a positive control for wounding and MeJA treatment. Loading of gels with RNA is indicated by ethidium bromide staining (lower panels). (b) GUS activity in three independent *promOBP2:GUS* lines 6 hours after spraying with 500 µM MeJA or wounding. Proteins were extracted from four 6-week-old treated plants each in three biological replicates. Transcript level was measured using Q-RTPCR for (c) MeJA treatment (d) wounding, and (e) herbivore feeding. (f) IGS were determined using HPLC after herbivore feeding. For herbivore feeding data are mean ± SD (n=3) and samples were replicated with individual plants from one experiment. For MeJA and wounding experiments, samples were taken from two independent (MeJA) or one (wounding) experiments pooled from four plants. All samples were leaf samples taken from plants prior to bolting. Asterisks (*) indicate values that are significantly different ($p < 0.05$) in comparison to respective controls.

3.3.4 *OBP2* expression pattern

Using semi-quantitative RT-PCR, *OBP2* was previously found to be expressed in all organs of six-week-old Arabidopsis plants, revealing the highest expression in roots and leaves, and a comparatively weak expression in stems and flowers (Kang and Singh, 2000). To investigate *OBP2* expression at the cell and tissue level ~1-kb of the 5´ upstream regulatory region of *OBP2* was fused to the *E. coli* β-glucuronidase (*GUS*) reporter gene and transferred to the nuclear genome of Arabidopsis. GUS activity was observed in the central cylinder (vascular tissue and pericycle) of both main and lateral roots, while it was absent from root hairs or the root cap (Figure 3b - d). In leaves, GUS expression in the vasculature was strongest in phloem cells (Figure 3e, f). GUS staining was also found in the vasculature of stems (Figure 3g, h), in stamen filaments of flowers, whereas weaker staining was detectable in the vasculature of petals and carpels (Figure 3i-l). GUS activity in the vascular tissue was observed from the seedling stage to the mature plant (Figure 3a - l).

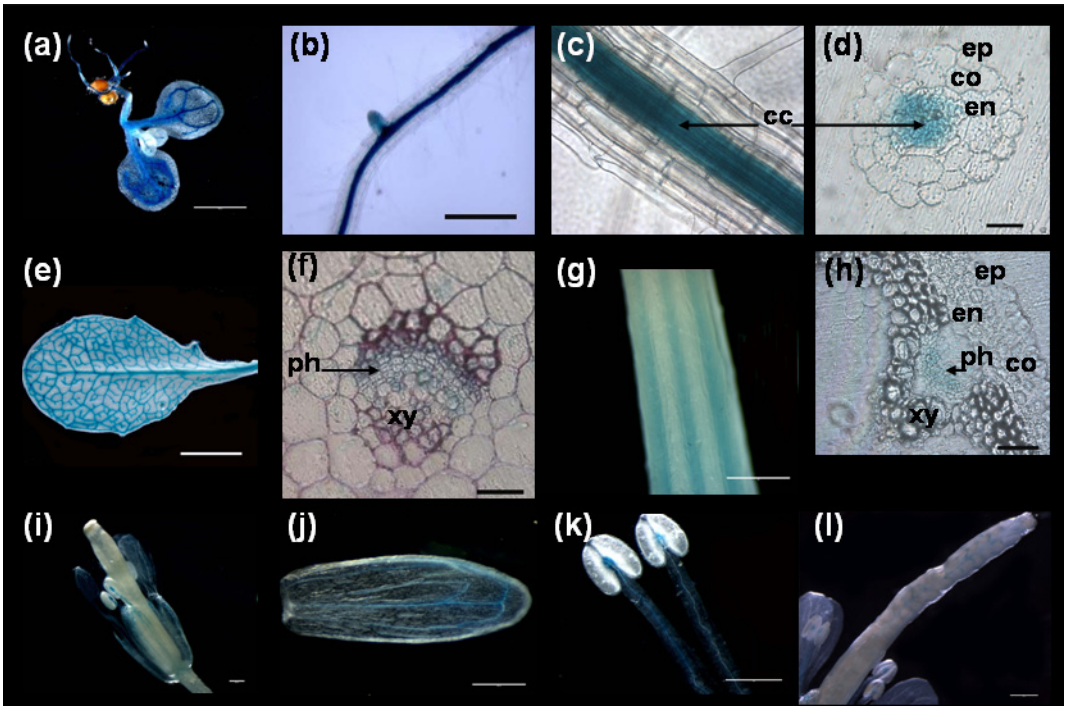

Figure 3. *OBP2* is expressed in the vasculature throughout the Arabidopsis plant. Plants were transformed with the *promOBP2:GUS* fusion construct. (a) Seedling kept for 5 days on sterile agar medium (bar, 5mm). (b) Root from 2-week-old plant (bar, 5mm). (c) Longitudinal view of root. (d) Root, transverse section (bar, 200µm). (e) Mature leaf (bar, 5mm). (f) Vascular bundle of a leaf, cross section (bar, 50µm). (g) Flower stalk, longitudinal view (bar, 5mm). Note the blue GUS staining along the vascular bundles. (h) Flower stalk, cross section through a vascular bundle (bar, 200µm). (i) Flower (bar, 0.5mm). (j) Petal (bar, 0.5mm). (k) Stamen (bar, 0.5mm). (l) Style (bar, 0.4mm). ph, phloem; xy, xylem; ep, epidermis; co, cortex; en, endodermis; cc, central cylinder.

3.3.5 Phenotypic effects of altered *OBP2* expression

Mature *35S:OBP2* plants exhibited a strong apical dominance, reduced overall height, and an increased number of curly, small rosette leaves; however, the total leaf number was slightly decreased compared with the wild type (Figure 4a-e). Flower morphology was not affected but flower number was reduced and fewer and smaller siliques were produced reducing seed yield. Under long day conditions *35S:OBP2* plants bolted four weeks later than wild-type plants but once the inflorescence was initiated no further retardation was observed (Figure 4c, d). To investigate the basis of reduced plant size we counted and measured leaf cells. In fully expanded third leaves of *35S:OBP2* plants epidermal and parenchyma cell size was significantly reduced and the cell number was significantly increased in comparison to the wild-type (Table 1, Figure 4f-i). Interestingly, there was an increased ratio of spongy to palisade parenchyma cells

compared to wild-type. This combined with the reduction of intracellular space in *35S:OBP2* leaves are likely to contribute to the observed upward leaf curvature. This leaf phenotype was also observed in *OBP2-GR* lines with DEX-inducible *OBP2* activation. Leaves started to curl 3-4 days after DEX application (Figure 4j, k). In addition, we found opposite effects in mature *RNAi-OBP2* plants which were slightly larger due to increased leaf size and had increased epidermal cell area (Table 1).

Seedlings of *35S:OBP2* lines had shorter hypocotyls and reduced number of lateral roots and this was also seen when *OBP2-GR* plants were germinated on DEX-containing media (Figure 5a, b). An opposite phenotype was seen in *RNAi-OBP2* lines which had longer hypocotyls and increased lateral root formation (Figure 5a).

Table 1. Analysis of epidermis, palisade and spongy cells of third rosette leaves of five-week-old wild-type (C24), *35S:OBP2* and *RNAi:OBP2* plants. Data for epidermis cells were obtained by analyzing dental resin imprints, whereas data on palisade and spongy cells were obtained from transverse sections of leaves embedded in Technovit. Data are mean ± SD (n=3-6) *Asterisks* (*) indicate values that are significantly different from the wild-type plants (Student´s t-test; $P < 0.05$).

Epidermal cells					
	C24 (n=6)	*OE*#8 (n=5)	*OE*#10(n=5)	*R#5* (n=5)	*R#11* (n=5)
	Adaxial epidermis				
Total cells[a]	470.4±72.2	599±25.7*	983± 124*	426±59	464±62
Cell area (μm^2)[b]	4327± 650	1581± 65*	1439± 72*	5041±720*	5127±673*
	Abaxial epidermis				
Total cells[a]	521± 43.8	622± 58.3*	990.1±157*	504±57	486±72
Cell area (μm^2)[b]	3915±348	1526±74*	1446± 70*	4728±634*	4812±578*

Palisade and spongy cells			
	C24 (n=3)	*OE*#8 (n=3)	*OE*#10(n=3)
Total cells[c]	367.8 ± 21.15	474.8 ± 26.2*	794.6 ± 24.3*
Palisade cells[a]	83.84 ± 7.32	89.53 ± 8	150.3 ± 8.65*
Spongy cells[a]	283.9 ± 13.83	385.2 ± 18.2*	644.3 ± 15.65*
spongy /palisade cells	3.38	4.3	4.28
Cell length[b]	53.25 ± 0.73	32.8 ± 0.81*	35.7 ± 1.87*
Cell width[b]	28.71 ± 0.83	16.9 ± 0.52 *	19.3 ± 0.66*

[a] Data are mean values ± SE from n plants.
[b] Data are mean values ± SE for ~100 cells from n plants.
[c] Total number of cells in the section, excluding epidermal cells, xylem and phloem cells.

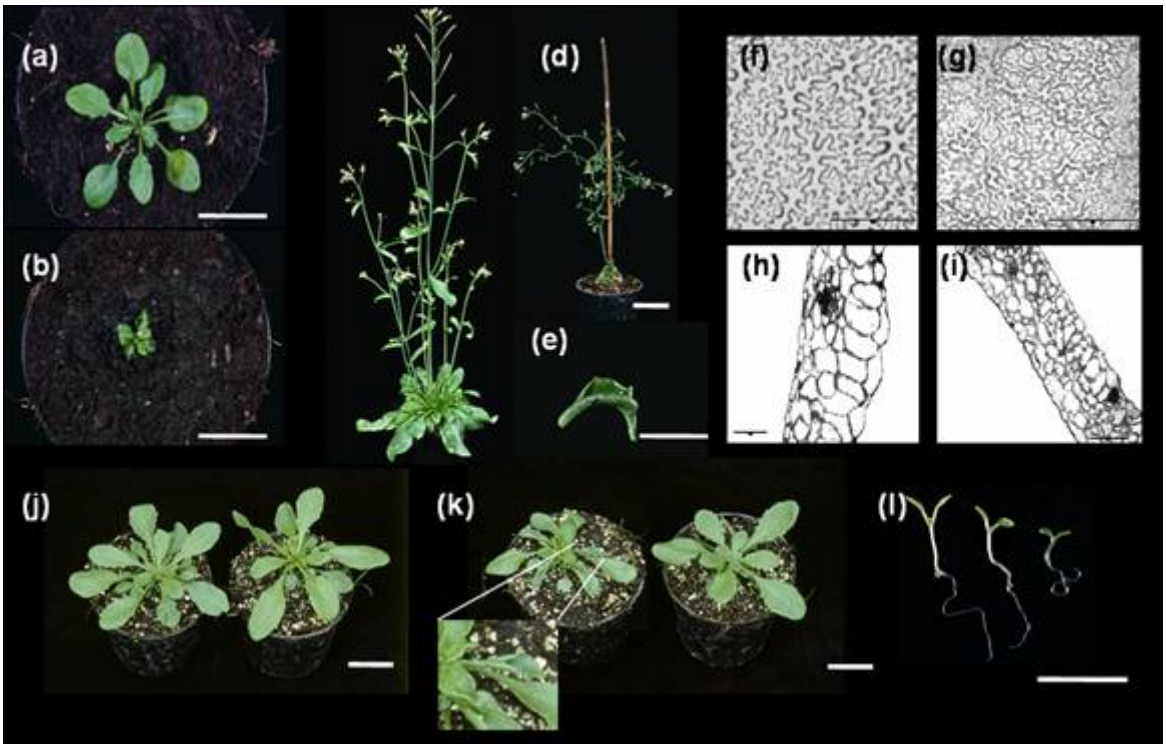

Figure 4. Phenotype of plants with modified *OBP2* expression

(a) Four-week-old wild-type plant (bar, 2cm). (b) Four-week-old *35S:OBP2* transgenic line (bar, 2cm). (c) Seven-week-old wild-type plant (bar, 3cm). (d) Twelve-week-old *35S:OBP2* plant (bar, 3cm). (e) Third mature leaf of *35S:OBP2* plant (bar, 0.5cm). (f-i) Cells of the third mature leaf of 5-week-old wild-type and *35S:OBP2* plants were microscopically analysed. (f) Adaxial epidermis of wild-type leaf (bar, 50µm). (g) Adaxial epidermis of *35S:OBP2* leaf (bar, 50µm). (h) Transverse section through the central part of a wild-type leaf (bar, 200µm). (i) Transverse section through the central part of a *35S:OBP2* leaf (bar, 200µm). (j) Five-week-old empty-vector control plants (d143) one week after DEX (left) or control (0.5% ethanol) treatment (right) (bar, 3cm). (k) Five-week-old *OBP2-GR* plants one week after DEX (left) or control (0.5% ethanol) treatment (right) (bar, 3cm). The magnification shows the newly forming, curly leaves that develop upon DEX treatment. (l) Seedlings of *RNAi:OBP2* (left), wild-type (middle), and *35S:OBP2* (right) plants kept on agar plates. Note the reduced hypocotyl length in case of the over-expression line (bar, 0.5cm).

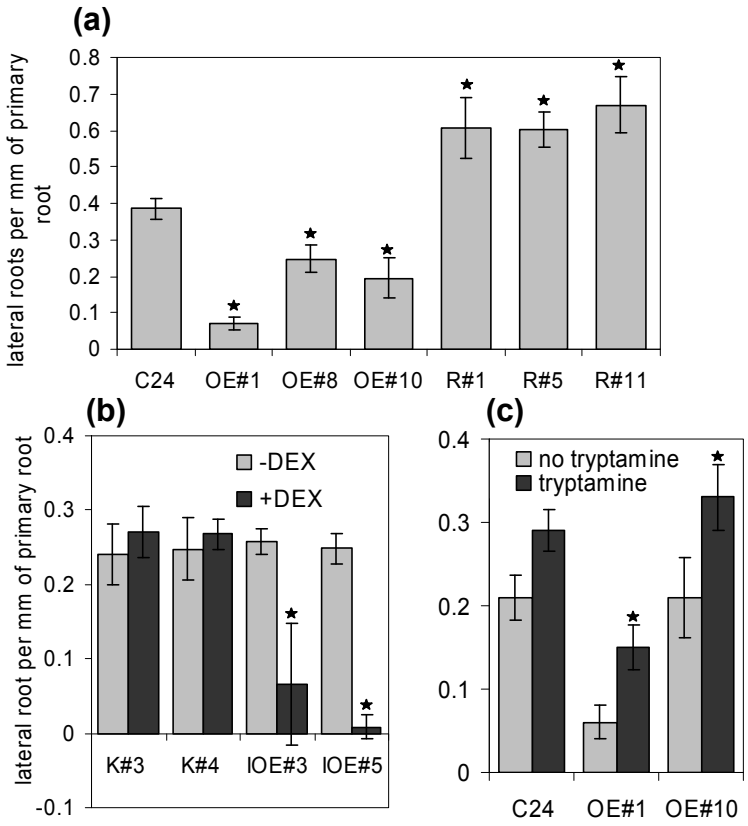

Figure 5. Analysis of lateral root formation in wild-type (C24), *35S:OBP2*, *OBP2-GR* and *RNAi-OBP2* lines. Given is the number of lateral roots produced per mm of primary root. Lateral roots were counted 12 days (a) or 10 days (b, c) after germination (DAG) and data are mean values ± SD (n=10-15). (a) Comparison of C24, *35S:OBP2* (OE#1, OE#8, OE#10) and *RNAi:OBP2* (R#1, R#5, R#11) lines. (b) Comparison of two empty-vector control lines (K#3, K#4) and two *OBP2-GR* lines (IOE#3, IOE#5) grown with (+) and without (-) DEX. (c) Quantitative analysis of lateral root formation in C24 and two different *35S:OBP2* lines (OE#1 and #10) grown with and without tryptamine. Asterisks (*) indicate values that are significantly different ($p < 0.05$) in comparison to respective controls.

3.3.6 Overlap between GS and auxin metabolism

IGS were shown to have dual role in auxin biosynthesis. IAOx is a precursor of both IGS and of IAA, and IGS themselves have been speculated to be source of the IAA precursor indole-3-acetonitrile (IAN) (Normanly and Bartel, 1999). It was previously shown that 0.1 µM 1-naphthaleneacetic acid (1-NAA) is sufficient to rescue lateral root development in the Arabidopsis *aux1* mutant (Marchant *et al*., 2002). This concentration of 1-NAA led to a highly increased number of lateral and adventitious roots in both wild-type and *35S:OBP2* plants so there was no remaining root phenotypic difference (data not shown). Lateral root formation in

35S:OBP2 lines was also restored by treatment with 100 µM tryptamine, an inhibitor of *CYP83B1*, which led to 2- to 3-fold more lateral roots per mm of primary root in comparison to plants grown in the absence of tryptamine (Figure 5c). However, in contrast to what these data might suggest free auxin was actually increased in *35S:OBP2* seedlings, which is also confirmed in seedlings super-transformed with an auxin-indicator *DR5-GFP* construct (Figure S2). Free and total auxin concentration was also elevated in mature leaves of *35S:OBP* plants but we measured no significant changes in seedlings or mature leaves of RNAi plants (Table 2).

Table 2. Auxin measurements. (A) Auxin level in fully developed wild-type (C24), 35S:OBP2, and RNAi:OBP2 plants. Data for total auxin content are mean ± SD (n=5). Data for free auxin are represented as x-fold. Each sample represents 35-40 pooled plants harvested in one biological experiment. (B) Free auxin level in liquid culture grown seedlings of wild-type (C24), empty-vector 35S control line (K#2), 35S:OBP2 and RNAi-OBP2 plants. Data for total auxin content are mean ± SD (n=3-4), no SD is indicated when n=2. Each sample represents a single flask. *Asterisks* (*) indicate values that are significantly different from the wild-type plants (Student´s t-test; $P < 0.05$).

A. Mature leaves				**B. Seedlings**			
RNAi	x-fold IAA[1]	*35S*	x-fold of IAA[1]	Total auxin[2]	*RNAi*	Free IAA	*35S*	Free IAA
#5	1.62*	#1	5.35*	172*±42	#5	810±65	#1	937*±146
#11	1.20	#8	5.77*	221*±16	#11	ND	#8	1158
#26	1.05	#10	9.11*	295*±88	#26	ND	#10	945*±173
C24	1	C24	1	105.6±7	C24	761±92	C24	761±92
K#2	ND	K#2	ND	ND	K#2	734±75	K#2	734±75

[1] Free IAA determined by GC/MS measurements
[2] Total auxin determined by ELISA of immunoaffinity purified samples

3.4 Discussion

OBP2, originally identified through its homology to OBP1, is a member of the Dof transcription factor family in Arabidopsis (Kang and Singh, 2000). We identified *OBP2* in a search for leaf expressed *DOF* genes. *OBP2* was previously reported to be most highly expressed in leaves and roots (Kang and Singh, 2000). Promoter-*GUS* studies showed that *OBP2* exhibits phloem-associated expression in all organs examined, including cotyledons, leaves, flower stalks, anther filaments, siliques and roots. This expression pattern is intriguing and it suggests that a common upstream transcription factor triggers *OBP2* expression in vascular strands at different developmental stages and in different tissues. The identification of *cis* element(s) and the transcriptional regulator(s) binding these will require further work.

To elucidate the functional role of OBP2 in Arabidopsis we constitutively over-expressed it and performed array-based analysis of transcripts in fully developed leaves. This revealed an induction of genes important for plant defense responses. Comparison of these genes with a study investigating the response to herbivore feeding and related treatments (Reymond *et al*., 2004) identified significant overlap. Of the 2964 genes present on both arrays, 45 were induced in the herbivore study and 37 were induced in *35S:OBP2* plants. Six genes were induced in both studies (*VSP2*, *CYP79B2*, galactinol synthase, putative lectin, myrosinase binding protein and *OPR3*), 10.7-fold more than would be expected by chance (p= 2.432 × 10^{-5}, Fisher exact test). Remarkably, four of these genes belong to a single expression cluster containing 26 genes shown to increase in response to insect feeding and MeJA treatment but not punctual wounding and to require COI1 for induction by *Pieris rapae*. *CYP83B1*, not present on the Affymetrix array but confirmed to increase by Q-RTPCR in our experiments (and northern blots; data not shown), also belongs to this expression cluster. These data argued for a role of OBP2 in the response to herbivore attack. Furthermore the mutual genes include four involved in GS metabolism, possibly regulated by OBP2. Targeted expression studies on GS biosynthesis showed *CYP79B2/B3, CYP83B1* and *MAM-1* were up-regulated in *35S:OBP2* plants. Consistent with these findings there was an accumulation of indole- and short chained aliphatic glucosinolates.

Transcript profiling and GS measurements of *35S:OBP2* plants reflect long-term changes in the steady–state mRNA levels resulting from permanent higher *OBP2* expression. To investigate if these changes were directly related to changes in *OBP2* expression we used DEX-inducible over-expression and RNAi of *OBP2*. Expression of *CYP83B1* was up-regulated in *OBP2-GR* transgenic lines 10h after induction and increased further at 24h. However, the expression of *ATR1, CYP79B2* and *CYP79B3* were not consistently changed. *CYP83B1* transcript level was also consistently decreased by RNAi of *OBP2* expression. Consistent with the report that 35S promoter-driven over-expression or null mutation of *CYP83B1* alone is not sufficient to change IGS levels in leaves harvested from the plants before bolting (Naur et al., 2003) there were no consistent changes in IGS in response to inducible over-expression or RNAi of *OBP2*. However, IGS levels were increased in an inducible line when the expression of *CYP79B2* and *CYP79B3* were also increased. Enhanced *CYP79B2* and *CYP79B3* expression was variable, sometimes occurring in control lines after DEX-treatment. This indicates that the primary effect of OBP2 is on *CYP83B1*, an enzyme that catalyses the first dedicated step of IGS biosynthesis. However, changes in IGS levels are dependent on preceding enzymes, CYP79B2 and CYP79B3, which are common controlled by the transcription factor ATR1 and in our experiments their expression changes were always co-regulated. Constitutive 35S promoter-driven expression of *ATR1* also

increases the expression of *CYP79B2, CYP79B3* and *CYP83B1* in seedlings and their expression is reduced, but still detected, in leaves of *atr1* null mutants (Celenza *et al.,* 2005). However, there is no effect of the null mutation on the expression of these genes at the seedling stage. There is complex regulation of this pathway as *cyp79B2/cyp79B3* double mutants have enhanced expression of *ATR1, CYP79B2, CYP79B3* and *CYP83B1*, whilst *cyp83B1* mutants have elevated expression of *ATR1, CYP79B2, CYP79B3* and mutant *CYP83B1*, and *ASA1* and *TSB1* which encode enzymes of tryptophan biosynthesis (Celenza *et al*., 2005). Although *ASA1* and *TSB1* expression are returned to near wild-type levels in *cyp83B1/atr1* double mutants the steady-state transcript levels of *CYP79B2, CYP79B3* and *CYP83B1* remains substantially elevated (Celenza *et al*., 2005). This provides clear evidence that these enzymes are regulated by factors additional to, and independent of, ATR1. Differential regulation of *ATR1* and *CYP83B1* has already been demonstrated following IAA treatment which up-regulates *CYP83B1* (Barlier *et al*., 2000) but down-regulates *ATR1* (Smolen and Bender, 2002). It is possible that increased *CYP83B1* expression in *35S:ATR1* plants is related to the elevated auxin levels found in these plants. In our inducible over-expression experiments, *CYP83B1* was induced independently of *ATR1* although *ATR1* was co-regulated with *CYP79B2* and *CYP79B3*. Independent regulation of *CYP79B2/B3* versus *CYP83B1* would allow more precise and separate regulation of the IGS and IAA biosynthetic pathways. CYP83B1 is a regulator of auxin production in Arabidopsis by controlling the flux of indole-3-acetaldoxime into IAA and IGS biosynthesis and as a branching enzyme CYP83B1 should be able to precisely respond to the plant status and as such requires tight regulation (Bak *et al.,* 2001). The induction of *ATR1* and *CYP79B2/B3* in *35S:OBP2* but not *OBP2-GR* plants may be explained by the higher expression in the constitutive plants. Alternatively, massive and sustained overexpression of transcription factors may have secondary effects causing stress that may affect gene expression. This would be consistent with the accumulation of stress-related transcripts and metabolites in *35S:OBP2* plants. This is a problem that cannot be excluded from any study using constitutive over-expression and here we report the regulation of a GS biosynthetic enzyme in the short term using an inducible expression system.

There is further evidence for a role of *OBP2* in biotic stress responses possibly as part of a network that regulates GS biosynthesis in Arabidopsis. The biosynthesis of GS appears to be phloem localised. *CYP79B2, CYP79B3, UGT74B1* and *IQD* genes (Mikkelsen *et al*., 2000; Glawischnig *et al*., 2004; Douglas Grubb *et al*., 2004; Levy *et al*., 2005) exhibit the same expression pattern in leaves of Arabidopsis plants transformed with promoter-reporter (*GUS*) gene constructs. All five genes are strongly expressed in the whole leaf vasculature, including

major and minor veins as well as free-ending veinlets extending into the intercostal leaf regions. The detailed expression pattern of the *CYP83B1* gene is not known. Recent analysis of the vasculature transcriptome using expression profiling also showed very high phloem expression of *OBP2*, *CYP79B2/B3* as well as *MAM-1*, *CYP79F1/F2* and *CYP83A1* (Zhao *et al*., 2005), with *CYP83B1*, but not *ATR1* being also more highly expressed in phloem than xylem. In addition, *OBP2* and *CYP79B2*, *CYP79B3*, and *CYP83B1* are all induced by the same spectrum of treatments, including wounding, MeJA and herbivore feeding. Expression of *OBP2* was induced after 6h of herbivore feeding while expression of *CYP83B1, CYP79B2* and *CYP79B3* increased after 8h. 2,4-dichlorophenoxyacetic acid (2,4-D) has been demonstrated to enhance expression of *OBP2* in Arabidopsis leaves (Kang and Singh, 2000). Similarly, *CYP83B1* transcription was shown to be induced by IAA (Barlier et al., 2000) Finally, *CYP83B1* contains 11 DOF-binding sites in its 1-kb upstream 5´ regulatory regions, respectively. The promoter of *CYP83A1*, which is not up-regulated by *OBP2*, contains only 5 potential DOF-binding sites and a random ~1-kb sequence would be predicted to have less than 4 of such elements (Kang *et al*., 2003). A working model for OBP2 action is depicted in Figure 6.

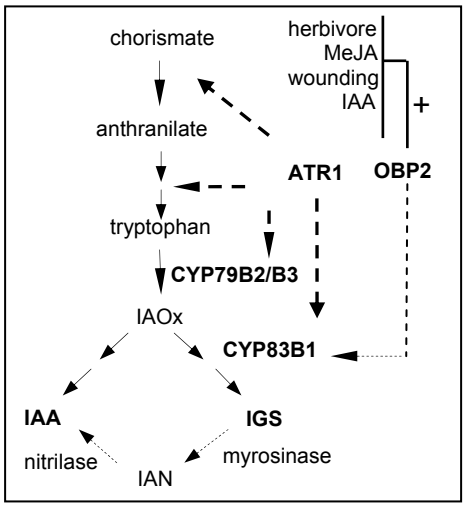

Figure 6. Proposed model of OBP2 function in plants. OBP2 is involved in biotic stress responses by regulating GS metabolism. Candidate downstream targets include the key enzyme of IGS synthesis, CYP83B1. Expression of *OBP2* is induced by herbivore feeding, MeJA treatment, wounding, and auxin.

In contrast to the clear role of OBP2 in GS metabolism and biotic stress responses, its role in the observed phenotypic changes is less clear. Certainly, it is likely that OBP2 regulates more genes than just *CYP83B1* and these may influence the phenotype of *35S:OBP2* plants. In addition, the relationship between these changes and possible changes in auxin metabolism is also uncertain, especially considering the redundancy and complexity of auxin biosynthesis and action. Increased IAA in mature leaves of *35S:OBP2* plants could result either from increased metabolic flow through IAOx due to enhanced *CYP79B2/B3* expression or the speculated role of

IGS as an additional source of the IAA precursor indole-3-acetonitrile (IAN) (Normanly and Bartel, 1999). Similar shoot phenotypes have been reported for *35S:CYP79B2* and *rnt1-1* plants, which have elevated auxin concentrations (Mikkelsen *et al.,* 2000; Bak *et al*., 2001). In seedlings the phenotypic data are not consistent with the measured increase in auxin although the restoration of lateral root growth by 1-NAA or tryptamine inhibition of CYP83B1 indicates that *35S:OBP2* plants have the capacity to respond to further increased auxin levels. The proximity of auxin to GS metabolism makes it an interesting candidate for study in relation to OBP2 function but the evident complexity and the interaction of auxin with many other signaling pathways put this beyond the scope of the present study.

It should be pointed out that in contrast to gain-of-function mutants of *CYP79B2/B3* and *CYP83B1*, plants over-expressing *OBP2* are characterised by up-regulation of both *CYP79B2/B3* and *CYP83B1* and an increase in both, indole and aliphatic GS. Although our inducible data indicate that not all of these changes are likely to be primary effects these plants may still be of considerable interest for studying the contribution of elevated GS levels to plant protection against pathogens.

3.5 Material and Methods

3.5.1 General methods

Standard molecular techniques were performed as described (Sambrook *et al*., 1989). Oligonucleotides were obtained from TibMolbiol (Berlin, Germany). DNA sequencing was performed by AGOWA (Berlin, Germany). Unless otherwise indicated, other chemicals were purchased from Roche, Merck (Darmstadt, Germany), or Sigma (Deisenhofen, Germany). For sequence analyses the tools provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), MIPS (http://mips.gsf.de/), and The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/) were used.

3.5.2 Plant material

Arabidopsis seeds were sown in soil (Einheitserde GS90; Gebrüder Patzer, Sinntal-Jossa, Germany) and grown in a growth chamber with a 16-h day length provided by fluorescent light at 80 or 120 µmol m^{-2} s^{-1} and a day/night temperature of 20/16°C and relative humidity of 60/75 %. In tissue culture, seedlings were grown in half-strength Murashige and Skoog medium (0.5 MS) supplemented with 1% sucrose and solidified with 0.7% agar under a 16-h day (140 µmol m^{-2} s^{-1}) / 8-h night regime (22°C). For root experiments, plants were grown vertically under the same

conditions except 1% agar was used. *Agrobacterium tumefaciens* strain GV3101 (pMP90) was used to transform *Arabidopsis thaliana* (L.) Heynh. cv. C24 (Clough, 1998). If not indicated differently measurements were performed on fully developed rosette leaves harvested from randomized 5- to 6-weeks-old plants before bolting.

3.5.3 Constructs

35S:OBP2: Polymerase chain reaction (PCR) was used to amplify the *OBP2* coding region using Arabidopsis C24 leaf cDNA as template. Primer sequences were as follows: *OBP2*-fwd., 5´-GTTTAAACATGGCGGAGAGAGCAAGGCAGG-3´ (added *Pme*I restriction site underlined); *OBP2*-rev., 5´-TTAATTAATTACCGGAGCGTCTGATAAACC-3´ (added *Pac*I restriction site underlined). The *OBP2* cDNA was inserted into pUni/V5-His-TOPO (Invitrogen, Karlsruhe, Germany) and, after sequence confirmation, cloned via the *Pme*I/*Pac*I sites into a modified pGreen0229 plant transformation vector (www.pgreen.ac.uk) containing a Cauliflower Mosaic Virus (CaMV) 35S promoter and *Pme*I/*Pac*I restriction sites. OBP2-GR: Primer sequences were as follows: *OBP2*-fwd., 5´-GGATCCATGGGTGGATCGATGGCGGAGAG-3´ (added *XbaI* restriction site underlined); *OBP2*-rev., 5´-GGATCCCACAAGAGATCATTAGAAGGACCC-3´ (added *XbaI* restriction site underlined). The *OBP2* cDNA was inserted into TOPO-TA (Invitrogen, Karlsruhe, Germany) and, after sequence confirmation, cloned via the *XbaI* sites into a d143 (pBI-GR) vector (Lloyd et al., 1994). *RNAi-OBP2*: A 139 bp 3'-UTR *OBP2* fragment was PCR amplified from a leaf cDNA library using the oligonucleotides RNAi-fwd 5´-CACCGATTGTGGTTTTACAACTTAAATTCG-3´ and RNAi-rev 5´-GGACTGTTACACACATGTATTGAGGC -3'. The PCR product was cloned into pENTR/D-TOPO of the Gateway System (). After sequencing, the *OBP2* fragment was transferred to the destination vector pJawohl8-RNAi (kindly provided by Dr. Imre Somssich, MPI for Plant Breeding, Cologne). Promoter-*GUS* fusion: A ~1-kb 5´ genomic fragment upstream of the ATG start codon was amplified by PCR using primers OBP2GUS-fwd (5´-AAGCTTAATTTGCTCCAATAATAACACATC-3´) and OBP2GUS-rev (5´-CCCGGGTGTTCCTTCTACCCTTTTTTTTTA-3´) from Arabidopsis C24 genomic DNA. The promoter fragment was inserted into plasmid pCR-Blunt II-TOPO (Invitrogen) and verified by sequencing. Subsequently, the promoter was fused to the *E. coli* β-glucuronidase (*GUS*) reporter gene in pGPTV-Kan (Becker *et al*., 1992), previously cut with *Hin*dIII and *Sma*I, resulting in plasmid *promOBP2:GUS*.

3.5.4 GUS assays

β-Glucuronidase activity was determined histochemically as described (Plesch *et al*., 2001) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as substrate (Jefferson *et al*., 1987). Fluorometric determination of β-glucuronidase activity was performed using 4-MUG (4-methylumbelliferyl β-D-glucuronide) substrate.

3.5.5 Induction experiments

Dexamethasone induction experiments: Soil grown plants were sprayed with 50µM DEX or control solution (0.5% ethanol) and harvested after indicated time. For plate experiments 5 µM DEX or 0.05% ethanol was added to the media.

MeJA and wounding experiments: Plants were sprayed with 500 µM MeJA, or control solution (0.006% ethanol) and mechanically wounded with tweezers.

Herbivore treatment: Eggs of *Spodoptera littoralis* were obtained from Syngenta Crop Protection AG (Stein, Switzerland). Larvae were kept on an artificial diet based on ground white beans at a temperature between 20°C and 22°C.Three 1-week-old larvae of *S. littoralis* were placed on each rosette of *A. thaliana* and allowed to feed for 24 h. Plants were covered with bread bags to prevent insects from escaping and placed in a controlled environment chamber with a 16 h light/8 h dark photoperiod, 70 % relative humidity, and a constant temperature of 22°C. Untreated control plants were kept under the same conditions. After 0.5, 1, 2, 4, 6, 8, 10, 24, and 72 h, six herbivore-damaged plants and six control plants were harvested for RNA extraction and GS analysis.

3.5.6 Anatomical analysis using light microscopy

To determine the number and area of epidermis cells, dental resin imprints were taken from the abaxial and adaxial leaf surfaces. Nail polish copies prepared from the imprints were analyzed by light microscopy. Data evaluation was based on imprints from five to six individual plants. For each plant, five separate fields of 0.31 mm^2 were analysed. Cross sections: Tissues were fixed in FAA solution (5% (v/v) acetic acid, 50% (v/v) ethanol, 3.7% formaldehyde (v/v)) and left overnight at room temperature. Dehydration was carried out through an ethanol series (50%, 70%, 90%, 95%, and 100%) at room temperature with 2h-4h per step. After dehydration the tissue was infiltrated for 2h in a solution containing 50% (v/v) ethanol and 50% (v/v) Technovit 7100. The samples were then incubated overnight in a solution of 1% Hardener I in 100% Technovit 7100. Polymerization was carried out by adding Hardener II. Sections of 4µm thickness were cut using histo-knives with a microtome and stained with 0.1% (w/v) toluidine

blue. Dental resin imprints and leaf cross sections were analyzed using the Olympus BX 41 System Microscope and Meta Value Software.

3.5.7 RNA gel blot analysis

Total RNA was prepared using TRIzol reagent (Gibco/BRL, Karlsruhe, Germany) according to the manufacturer´s instructions. RNA gel blot analysis was performed as described in Gomez-Merino *et al*. (2004).

3.5.8 Expression profiling

Two *35S:OBP2* lines were analyzed using Affymetrix GeneChips representing ~8.200 Arabidopsis genes. Total RNA was isolated from fully developed rosette leaves harvested from individual plants of two independent *35S:OBP2* lines, two different wild-type plants, and two independent empty-vector lines plants. Quality-checked RNA was sent to the German Resource Center for Genomic Research (RZPD, Berlin, Germany) for probe preparation and Arabidopsis GeneChip hybridisation (Affymetrix, Santa Clara, CA). Twenty µg of total RNA was used for double-strand cDNA synthesis (SuperScript Choice system, Gibco/BRL). Biotin-labelled cRNA was synthesized using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY). Affymetrix GeneChip experiments including washing and scanning procedures were performed as described in the Affymetrix technical manual. For each micro-array, overall intensity normalization for the entire probe sets was performed using MicroArray Suite software 5.0. Chip files were generated using the program's default parameters. Pair-wise comparison between the files of the *35S:OBP2* lines, two different control lines, and two wild-type plants was carried out. Genes were considered as altered when the signal was >2-fold changed, had an appropriate present call and was defined as increase or decrease by the software. Only genes that met these criteria in all eight comparisons (*35S:OBP2* line 8 x WT 18; *35S:OBP2* line 8 x WT 27; *35S:OBP2* line 8 x CTRL 11; *35S:OBP2* line 8 x CTRL 24; *35S:OBP2* line 10 x WT 18; *35S:OBP2* line 10 x WT 27; *35S:OBP2* line 10 x CTRL 11; *35S:OBP2* line 10 x CTRL 24; WT representing untransformed wild-type plants, and CTRL representing vector-alone transformed transgenic lines) were considered further.

3.5.9 Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent as described above. RNA (1 µg) was then reverse transcribed with Superscript II reverse transcriptase (Invitrogen) in a reaction volume of 20 µl to generate first-strand cDNA. Every cDNA preparation was tested for contamination with

genomic DNA by PCR with primers LEH−fw (5´-AACAGCAACAACAATGCAACTACTGATT-3´) and LEH−rev (5´-ACAAACAGAGACAAGAGACAAGACATGG-3´) that span an intron of the *LATE ELONGATED HYPOCOTYL* gene. Occasionally, genomic PCR products appeared after 35 amplification cycles, such samples were discarded. Real-time RT-PCR was performed with 1 µl of a 1:2 dilution of the first-strand cDNA reaction and SYBR Green reagent (Applied Biosystems, Foster City, CA), in a 20-µl volume, on a Perkin Elmer Geneamp 7300 machine, with the following primer pairs. *ACTIN2*: actin-fw (5´-ATGGCTGAGGCTGATGATATTCAAC-3´) and actin-rev (5´-TACAAGGAGAGAAC AGCTTGGATG-3´); *UBQ10*: UBQ-fw (5´-ATGCAGATCTTTGTTAAGACTCTCAC-3´) and UBQ-rev (5´-ATAGTCTTTCCGGTGAGAGTCTTC-3´); OBP2-fw (5´-GCATCCGTTGGATCTTTGAGC-3´) and OBP2-rev (5´-AAAGCGTATAGCCCCGTCGTT-3´); CYP79B2-fw (5'-TTTGATGGATTGTCTGGCGC-3') and CYP79B2-rev (5'-CAAAGACGAACAAGGCAACC-3'); CYP79B3-fw (5'-CGGTTTGTTTATCATCTCCGC-3') and CYP79B3-rev (5'-TTGCTTACCGCTGATGAAATC-3'); CYP83B1-fw (5' TCCGACCTTTTCCCTTATTTCG-3') and CYP83B1-rev (5'TTGAGACGTGCACTGAGACCAG-3'); ATR1-fw (5'-CGGGTCTTAAGTAATTAGCC-3'); ATR1-rev (5´-AAGAAAGGAGCTTGGACTCC-3´); MAM1-fw (5-CATGTTGCTCTTCTGTGTCC-3') and MAM1-rev (5'-ACATACCGAACAAGCTTCCC-3').

Data were normalized to *ACTIN2*; nCT = CTgene – CT*ACTIN2*; CT refers to the number of cycles at which SYBR Green fluorescence in a PCR reaches an arbitrary value during the exponential phase of DNA amplification, set at 0.3 in all experiments, and then compared according to the formula, Cr (change in signal log ratio) = nCTcontrol – nCTsample. The use of *UBQ10* control gene did not affect the results, therefore, only the *ACTIN2* data are presented.

3.5.10 Metabolite profiling

Leaves (60 mg fresh weight) of eight individual plants were frozen in liquid nitrogen and powdered in a Retsch mill. Metabolite extraction and measurement were performed as described before (Roessner *et al*., 2000). For data analysis a retention time and mass spectral library for automatic peak quantification of metabolite derivatives was implemented within the MASSLAB method format. The t-tests were performed using the algorithm incorporated into Microsoft EXCEL (Microsoft Corporation, Seattle, WA, USA). The word ´significant´ is used in the text when the change in question has been confirmed to be statistically significant ($P < 0.01$) with the t-test.

3.5.11 Glucosinolate measurements

Leaves were harvested, frozen in liquid nitrogen and lyophilized to dryness. GS analysis was performed as described before (Brown *et al*., 2003), with the following modifications: metabolites were extracted with 80% (v/v) methanol, and 4-hydroxybenzyl-glucosinolate was used as internal standard.

3.5.12 Hormone measurements

Leaves were harvested and frozen in liquid nitrogen. 2H5 – indole-3-acetic acid (Sigma, Germany) was used as internal standard. Samples were extracted with 5ml of Bieleski solvent for 30 min at 70°C and than were passed through C18-u columns, preconditioned with methanol and Bieleski solvent, subsequently. Eluates were dried under vacuum, re-dissolved in diethylether and successively applied to a aminopropyl cartridge (preconditioned in diethylether), then washed with chloroform:2-propanol 2:1 (v/v) and eluted with 10% formic acid in diethylether (Mueller *et al*., 2002). Resulting eluates were dried and re-dissolved in water pH 2-3 (HCl) and applied to ENV+ columns (preconditioned with water, pH3). Elution was carried out with water pH 7 followed by 0.35 M hydroxylamine in 60% methanol / water (v/v) (Dobrev *et al*., 2002). Combined eluates from both steps were evaporised and then derivatised with N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) according to Birkemeyer *et al*. (2003). Derivatised samples were measured on an ion trap system (Saturn 2000, Varian inc. Palo Alto, USA) in EI-MRM-Modus using the following parameters: 30 m DB 5-MS fused-silica column with 0.25 mm inner diameter, 0.25 µm film thickness (Agilent Technologies); injection temperature: 230°C; injection volume: 1 µl, splitless mode; carrier gas: helium, flow: 1 ml / min; temperature program: 5 min at 70°C, then raising with 10°C / min to 200°C, with 15°C / min to 310°C, hold 10 min; transfer line temperature: 250 °C; ion trap temperature: 200°C; parent ions: 232,234 and excitation amplitudes: 0.60.

We also determined auxin using an alternative method. Plant material was processed as described by Prinsen *et al*. (2000). After the methylation with diazomethane the samples were dried under a nitrogen stream, dissolved in a mixture of 50 µl ethanol (70%, v/v) and 450 µl of phosphate buffer (50 mM, pH 7.2) and subjected to an auxin-specific immunoaffinity extraction almost identical, in terms of its execution, to that for cytokinins (Novák *et al*., 2003). The final analysis was done either by a competitive ELISA (Strnad, 1996) using anti-C1-IAA antibodies and IAA-alkaline phosphatase tracer. The assay was calibrated using [^{3}H]IAA.

3.5.12 AGI code numbers

The AGI code numbers for the genes tested in this study are as follows: At1g07640 (OBP2); At4g39950 (CYP79B2); At2g22330 (CYP79B3); At4g31500 (CYP83B1/SUR2); At5g60890 (ATR1); At1g01060 (LATE ELONGATED HYPOCOTYL); At3g18780 (ACTIN2); At4g05320 (UBQ10); At5g44420 (PDF1.2); At5g23010 (MAM-1).

3.6 Acknowledgements

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3.7 OBP2 – data obtained after publication of the paper Skirycz *et al.,* (2006)

3.7.1 *CYP83B1* is a direct target of OBP2

Previous work revealed that OBP2 regulates expression of *CYP83B1* (Skirycz *et al.,* 2006). To establish whether *CYP83B1* is a direct target of OBP2 chromatin immuno-precipitation (ChIP) experiments were performed. For this purpose transgenic plants expressing *GFP-OBP2* fusion protein under control of the *OBP2* promoter in the *OBP2* knock-out background were generated (Figure 7a). Several transgenic lines (#5 and #6) were selected using Q-RTPCR (Figure 7d) and as expected GFP-OBP2 fusion protein localised in the nuclei of phloem cells (Figure 7b-7c).

Enrichment of *CYP83B1* promoter fragments spanning approximately 300bp between ~900bp and ~1.2kb upstream of the translational initiation codon was detected in OBP2-GFP lines versus control plants indicating that OBP2 directly regulates *CYP83B1* expression. Enriched fragments contain several DOF binding sites, two in tandem repeat. Further experiments are necessary to establish which of these sites are functional.

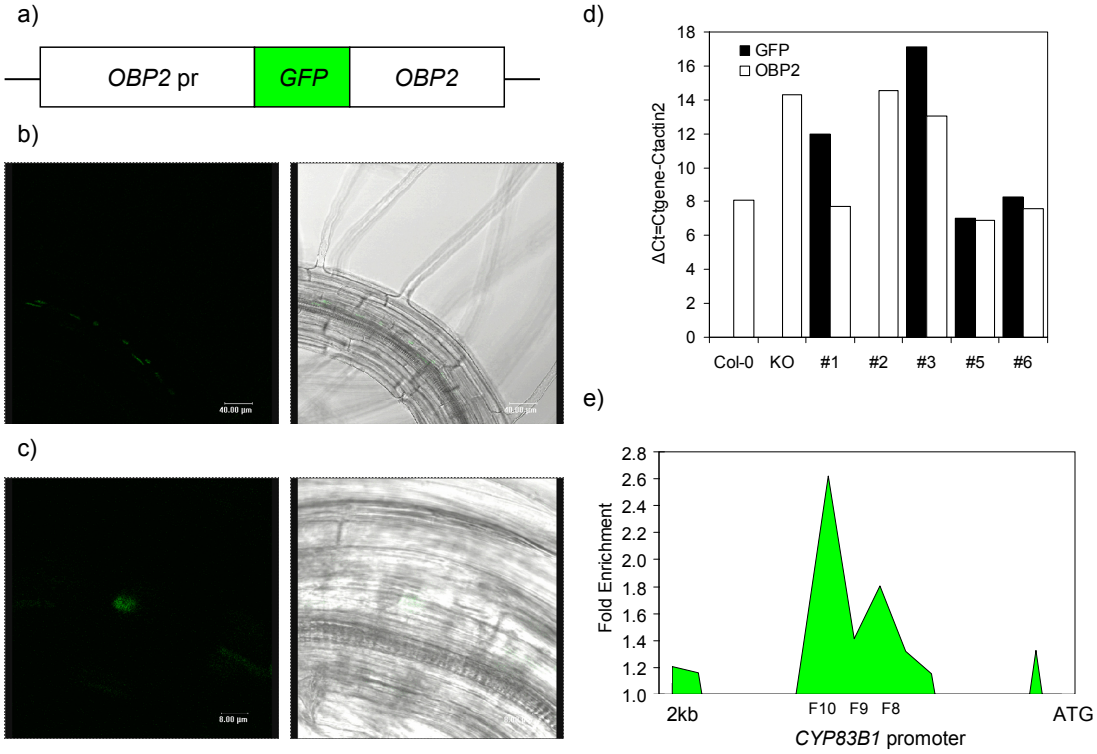

Figure 7. OBP2 binds to *CYP83B1* promoter. a) Schematic representation of *OBP2promoter:GFP-OBP2* construct. b, c) Presence of GFP-OBP2 protein was validated by fluorescent microscopy (Olympus BX51). As expected GFP signal was observed in the nuclei of phloem cells. Photos depict roots of week old seedlings, (bar=40μm). d) Transcript level of *GFP-OBP2* and *OBP2* was determined by Q-RTPCR in

wild type (Col-0), OBP2 knock-out (KO) and several *OBP2promoter:GFP-OBP2* plant lines (#1-#6). e) ChIP was performed as described by Leibfried *et al.,* 2005 using anti-GFP antibody. Graph depicts fold enrichment along 2kb of *CYP83B1* promoter measured by Q-PCR and overlapping primer pairs (F1-F16); each pair amplifies ~100-150bp long fragment. Data were normalised to input and genomic fragment of heat shock protein 1. Control plants expressing GFP fused to TF CONSTANS under control of CONSTANS or SUCROSE2 promoter were kindly obtained from Prof. Georg Coupland (MPI for Plant Breeding, Cologne).

3.7.2 Material and Methods

OBP2 knock-out lines: Seeds of *OBP2* T-DNA insertion line SALK_143145.56.00.x were obtained from the Nottingham stock centre. Homozygous plants were selected using PCR whilst *OBP2* transcript level was investigated using Q-RTPCR. The T-DNA insertion site lies in the 3'UTR, 305bp downstream of the stop codon. ***OBP2promoter:GFP-OBP2*** **construct:** The *OBP2* promoter was amplified from genomic DNA using following primers: CCGCGGCCAATAATAACACATCCATCTTTC, GCGGCCGCTGTTCCTTCTACCCTTTTTTTTT, the *OBP2* gene was amplified from the leaf cDNA using following primers: AAGCTTGGTGGATCGATGGCGGAGAGAG, GTTTAAACTCACAAGAGATCATTAGAAGGAC. Underlined are restriction sites. PCR products were inserted in TOPO-TA vector (Invitrogen, Karlsruhe, Germany) and subsequently cloned into modified pGreen-GFP vector: GFP gene was cloned into pGreen from the entry vector kindly provided by the group of Prof. Philip Benfey (Duke University) using *NotI* and *HindIII* restriction sites. **ChIP:** Chromatin immuno-preciptation and data analysis was performed as described by Leibfried *et al.,* (2005). Anti-GFP antibody (Abcam, Cambridge, UK) was used at concentration of 1ug per sample. Primers used for Q-RTPCR are given below. Additionally they are underlined in the *CYP83B1* promoter sequence; with green indicated ATG, with red DOF binding sites, with blue fragment enriched in ChIP experiments.

		Forward	Reverse
F1	*CYP83B1 promoter*	CGGCTATAATCAATAAGAGATCC	CTGTCACCTGTCAAAAAATCC
F2	*CYP83B1 promoter*	GGATTTTTTGACAGGTGACAG	CAACTCTTCATCGATCTCGC
F3	*CYP83B1 promoter*	GCGAGATCGATGAAGAGTTG	TCAAGAATGATGTCTAAATGAAC
F4	*CYP83B1 promoter*	GTTCATTTAGACATCATTCTTGA	GCATAGGAGAAAATGATCGCT
F5	*CYP83B1 promoter*	AGCGATCATTTTCTCCTATGC	CTCACTGTGACCTACTGTTC
F6	*CYP83B1 promoter*	GAACAGTAGGTCACAGTGAG	GGGACATCTTTAGGCTCGTT
F7	*CYP83B1 promoter*	AACGAGCCTAAAGATGTCCC	TGTCACACTTCTTGTCAACC
F8	*CYP83B1 promoter*	AGGTTGACAAGAAGTGTGAC	CGTTGACAAATAAGTTGGAGG
F9	*CYP83B1 promoter*	CCTCCAACTTATTTGTCAACG	GAATCTGACAACGAGGATCAT
F10	*CYP83B1 promoter*	CAGCTCTTAGTCTGAGGAAC	TTCTATGCCGTTGCTTGCAT
F11	*CYP83B1 promoter*	ATGCAAGCAACGGCATAGAA	GTTTGGGAATTTAATGGAAGC

F12	*CYP83B1 promoter*	GCTTCCATTAAATTCCCAAAC	TGTTTCTTACTTATTAGATTCTCAG
F13	*CYP83B1 promoter*	CTGAGAATCTAATAAGTAAGAAACA	CTAATTATTCTGTTATCTACACCTG
F14	*CYP83B1 promoter*	CAGGTGTAGATAACAGAATAATTAG	GGAGAAACCAGCGAAAACGA
F15	*CYP83B1 promoter*	TCGTTTTCGCTGGTTTCTCC	GGTTCTACGTTTAGGGATCG
F16	*CYP83B1 promoter*	CGATCCCTAAACGTAGAACC	TGACAGCTGAGCTCGATTTG
HSF1	*HSF1 promoter*	GCTATCCACAGGTTAGATAAAGGAG	GAGAAAGATTGTGTGAGAATGAAA

C**CGGCTATAATCAATAAGAGATC**CATttttttctgtttgacttacttaagtctttgtgttcggatACTTTTCTACTTTGAATGATGATGTGGTTTATAT
AGCAAGACAAAAAGTTTTATTGGTTGGTTTGTTTT**GGATTTTTTGACAGGTGACAG**AATTTTCCCTTGTTTTTAAAACGTGAT
GTTAGTAGGTGGACTACTGGTTTTTGGAATTAAAAAATGTAACTTGGGTTACACAATAATACTGAAAG**CGAGATCGATGAAG**
AGTTGAAAATGTGAAGATATTGGATAAGAATATAACCTCTTGGTTTTGTTTTCTAAAAATATACTATAGAACAGTAAGTATATT
ATGAAGTAATAAAATATCATCAACTTGTTTTTTATAAATCTCATAAACTTTT**GTTCATTTAGACATCATTCTTGA**TTTTTTTCTTT
TTGAACTGATATGATCGATTTAAAAACAAAGCATAAAAAAATGGTACTTTAGAAAAATTCCTAAAAATACCTATGAAATTTGA**AG**
CGATCATTTTCTCCTATGCTATAACGTAAACTGAAAGAAAAAAAAAGTATACGTAACCAAAAAACTAAACTCTCAGTTTTTAA
GTTTTCTAATTACTCTGTAGTCAATTTTTGTAGAAAAATATGTTCATATTTTTGCAGTT**GAACAGTAGGTCACAGTGAG**ACAA
ATATTCTACTTCGTCAATCTAAGATGAATCGTACATAAATTCAACTCGGTAGAGAAAAAAAAAAAAGAGTATTTCGGAG**AACG**
AGCCTAAAGATGTCCCTATTGACTTCGCAGCCGCTATTGAAATTACTAAGCCGTCAATATTAACGACTTTGTTCTCATGCGG
ACATTAAAATTTATAGAACCAATCTCATGGGCTTACTAACAAATGTTA**AGGTTGACAAGAAGTGTGAC**AAAAAAAATAAAAA
GTCTACTTCGTTGAGAAAATTCAAAATATACAAACAGTCAAATTAATTTATTACAATTACACGTAAATTGGTTTCTGTCAAATC
TT**CCTCCAACTTATTTGTCAACG**AAACTTTATCTACTCTTGGACAAAAGAAGAAGAATGATCATCGATCATCGATCATCATCA
TCTTGAAAACTAGTTAAGCTAAAA**CAGCTCTTAGTCTGAGGAAC**CATAAAATCTCTATGGTTCGTCATCTTCATCCTCTTCTC
TTTCAGCTTACTAGTTTCATCATCACTGCTACTATCATCATCATCATCATCATTGAGTTTATGATCCTCGTTGTCAGATTC
TTGCA**ATGCAAGCAACGGCATAGAA**TTAGGGTTAATTTTAGTGTAGATGCTGAAAGAAGACAAGGACGATCTTCTTCTTAG
CTTATTAGCAATAAGCAATCTTCTTCTCTTGTTCAATGGACTCTCCACTTTCACCAAATCGTTTGTGTTACTC**GCTTCCATTAA**
ATTCCCAAACGATTTCGATTTCCCTATGTAATGGTTTGATAATCCTCTCCTGTAACAAAAACCAAAACCTAAACCACATGACT
AAACAAAAACACAACATGTAGTAATAACATTTAAA**CTGAGAATCTAATAAGTAAGAAACA**ATTTCAACATCTTACAGATTCAT
CAATAAAATCAAACCCAAGAACATAAGAGAATCTAATGATTTACTTTATTCTTGTAATAAAATAAATCGATCGAATATATT**CAG**
GTGTAGATAACAGAATAATTAGAAGATAATTAATAAAGAAACGTACTTAATCGGAAGAGAATCTTCCAAAGATGAACTAAAC
GAATTGAGCCATCTTCCTTGTGATGACGAAACGGCGTCGTCTTCATCTTCC**TCGTTTTCGCTGGTTTCTCC**AACGGACGACG
AACTCTCCGGCGGAGGAGATCTACCACATCTCCTCAAACCAATCCGGCTAGACAGAGACGCCGGTACGGCCTGATCATGC
GTCGTGACGCTGCTA**CGATCCCTAAACGTAGAACC**TACCAGAACCTCCATTGATTCCGGTAGAATTCTTGAAGCCTTTGTT
TATCAACCAAATCGAGCTCAGCTGTCATAAAATCGGAGCATCCGCGTAGGTTAAGAGCTC

4. Transcription factor AtDOF4;2 affects phenylpropanoid metabolism in *Arabidopsis thaliana*

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Authors´ contributions

Bernd Mueller-Roeber conceived the idea for the study. The research was designed and planned by Bernd Mueller-Roeber and Aleksandra Skirycz. The experimental work was done by Aleksandra Skirycz. The research was supported through collaborations as follows: flavonoid measurements were performed in collaboration with Dorota Muth and Maciej Stobiecki, and *RNAi-DOF4;2, 35S-DOF4;2 and AtDOF4;2-GUS* constructs were prepared by Maria-Inès Zanor and Isabell Witt. Szymon Jȯzefczuk assisted as a student worker in the data collection; he was supervised by Aleksandra Skirycz.

4.1 Abstract

- In a phenotypic screen of plants constitutively over-expressing DOF (DNA-binding-with-one-finger) transcription factors under the control of the Cauliflower Mosaic Virus 35S promoter we identified *AtDOF4;2* as a gene inducing a bushy plant phenotype and potentially being involved in the regulation of phenylpropanoid metabolism in Arabidopsis.
- Further molecular and biochemical characterisation was performed in parallel using transgenic plants with enhanced and reduced *AtDOF4;2* expression. The expression pattern of *AtDOF4;2* was determined by quantitative real-time polymerase chain reaction (Q-RTPCR) and through promoter-β-glucuronidase (*GUS*) fusions, indicating preferential transcriptional activity in axillary buds of the flower stalk, the hypocotyls periderm and in tapetum cells.
- Constitutive over-expression and RNAi-mediated silencing of *AtDOF4;2* caused reciprocal changes in the expression of flavonoid biosynthetic genes and the accumulation of flavonoids under cold and high-light conditions. Moreover, tapetum specific over-expression of *AtDOF4;2* led to pollen grains devoid of flavonols. In contrast to its negative influence on flavonoid biosynthesis and coincident with high expression in the periderm and tapetum, AtDOF4;2 positively influences the production of hydroxycinnamic acids in the hypocotyl and flower buds implicating its possible importance for suberin and sporopollenin production.
- These data provide evidence that AtDOF4;2, influences phenylpropanoid metabolism in an environmental and tissue-specific manner.

4.2 Introduction

Phenylpropanoids are important secondary metabolites which are involved in numerous biological processes. Their diversified functions reflect their structural diversity, with for example more than 8,000 different flavonoids identified in vascular plants (Pietta, 2000). Flavonol aglycons are highly active and were shown to have important developmental functions both as direct inhibitors of auxin transport (Jacobs & Rubery, 1988; Murphy *et al.*, 2000; Brown *et al.*, 2001; Buer & Muday, 2004; Peer *et al.*, 2004) and as signals exported from legume roots and sensed by bacteria, a prerequisite for nodule formation (Long, 1989; Mathesius *et al.*, 1998; Perret *et al.*, 1999; Kobayashi *et al.*, 2004; Novak *et al.*, 2004). Glycosylation confers solubility and vacuole stored glycosylated flavonoids were demonstrated to be involved in plant protection against UV and visible light (Li *et al.*, 1993; Gould *et al.*, 1995; Landry *et al.*, 1995; Havaux and Kloppstech, 2001). Lignins and hydroxycinnamic acids, other phenylpropanoid compounds, are embedded in the cell walls as a part of complex biopolymers having both protective and

structural roles in specialized cells such as xylem, periderm or pollen grains (Piffanelli *et al.*, 1998; Rogers & Campbell, 2004; Scott *et al.*, 2004; Franke *et al.*, 2005).

Phenylpropanoids are synthesized via well-characterized biosynthetic pathways (Figure 1) and the production of different classes of phenylpropanoid compounds is regulated mainly on the transcriptional level by both environmental and developmental stimuli. Genes that are specific for different branches of phenylpropanoid metabolism are highly co-expressed and regulated by the combination of different transcription factors (Gachon *et al.*, 2005). So far the best understood is the interplay between transcription factors (TFs) of the R2R3-MYB and basic helix-loop-helix (bHLH) families. It was first described in *Zea mays* where the interaction of C1 (MYB) and R (bHLH) TFs is necessary for the activation of anthocyanin biosynthesis (Goff *et al.*, 1992). In seeds of Arabidopsis, the MYB transcription factor TRANSPARENT TESTA2 (TT2) forms a complex with the bHLH transcription factor TT8 and a WD40 scaffold protein to control the expression of *BANYULS*, a proanthocyanidin biosynthetic gene. In leaves, TT8 and it closest homolog GLABRA (GL3) and ENHANCED GLABRA (EGL3) control anthocyanin biosynthesis through interactions with two homologous MYB proteins PRODUCTION OF ANTHOCYANIN PIGMENTS 1 (PAP1) and PAP2 (Baudry *et al.*, 2004; Zimmermann *et al.*, 2004). Ectopic expression of *PAP1* and *PAP2* leads to anthocyanin, flavonol, and hydroxycinnamic acid accumulation (Borevitz *et al.*, 2000). *PAP1* was shown to be responsible for sucrose specific anthocyanin accumulation (Teng *et al.*, 2005) whilst *PAP2* is cold induced and was identified among genes positively correlating with acclimated freezing tolerance (Hannah *et al.*, 2006). Both *PAP1* and *PAP2* are also responsive to nitrate starvation and jasmonic acid treatment (Devoto *et al.*, 2005; Lea *et al.*, 2006). Although flavonoid biosynthetic genes are induced in *PAP1* over-expressers, there is no direct evidence of PAP1 and PAP2 binding to the promoters of these genes (Borevitz *et al.*, 2000; Tohge *et al.*, 2005). MYB TFs may also act independently of bHLH TFs, as demonstrated for the activation of chalcone and flavonol synthase promoters by MYB12 in co-transfection assays (Mehrtens *et al.*, 2005). In contrast to these positive regulators, MYB4 is a negative regulator of coumarate-4-hydroxylase and sinapate ester biosynthesis (Hemm *et al.*, 2001; Jin *et al.*, 2000). Its homologs from *Antirrhinum majus*, AmMYB308 and AmMYB330, repress hydroxycinnamic and monolignol biosynthesis when over-expressed in tobacco (Tamagnone *et al.*, 1998).

Recently microarray analysis of mutants with ectopic lignification phenotypes and dark grown, sucrose-supplemented, wild-type plants suggested that changes in the expression of specific members of the R2R3-MYB and DOF transcription factor families may have both positive and negative roles in the control of lignification (Rogers *et al.*, 2005). This is interesting as an

interaction between MYB and DOF transcription factors was previously demonstrated (Diaz *et al.*, 2002; Isabel-LaMoneda *et al.*, 2003).

Our work provides evidence for the involvement of a DOF family TF in phenylpropanoid metabolism. In *Arabidopsis thaliana*, *AtDOF4;2* negatively affects flavonoid accumulation under stress and its over-expression in the tapetum leads to the formation of flavonol-free pollen grains. In addition, consistent with high *AtDOF4;2* expression in the tapetum of flower buds and the periderm of hypocotyls, genes involved in hydroxycinnamate acid synthesis are induced in these tissues in *AtDOF4;2* over-expressing plants indicating *AtDOF4;2* may influence sporopollenin and suberin synthesis.

4.3 Results

4.3.1 Bushy phenotype of *35S-AtDOF4;2* plants

During a screen of plants constitutively over-expressing DOF transcription factors under the control of the Cauliflower Mosaic Virus 35S promoter we identified *AtDOF4;2* as potentially important for shoot branching in Arabidopsis. Whilst there were no visible morphological differences between RNAi-blocked *AtDOF4;2* (*RNAi-AtDOF4;2*) and wild-type plants, *AtDOF4;2* over-expressers were characterized by a bushy phenotype (Figure 2a). The main inflorescence of the *35S-AtDOF4;2* plants grew much slower compared with that of the wild-type. Although the number of axillary meristems was not visibly changed, the number and length of secondary inflorescences that emerged from rosette leaf axils was increased resulting in more buds and flowers (Figure 2b). Other phenotypic characteristics of *35S-AtDOF4;2* plants included delayed silique formation and greatly reduced seed numbers (Figure 2c). Manual-pollination experiments showed no difference in the pollen fertility with identical seed set when wild-type plants were pollinated with either wild-type or transgenic pollen (data not shown).

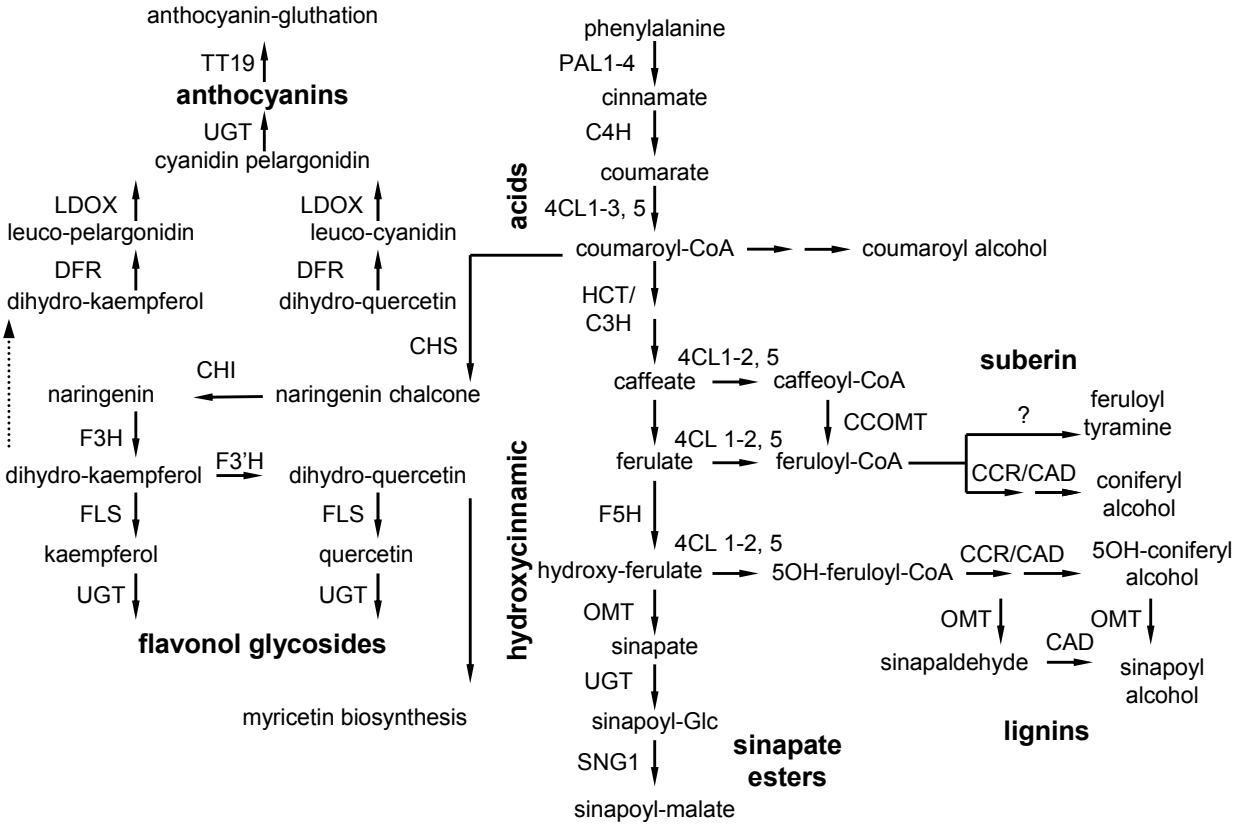

Figure 1. Schematic representation of phenylpropanoid metabolism. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavonol-3-hydroxylase; F3´H, flavonol-3´-hydroxylase; FLS, flavonol synthase; UGT, glucosyltransferase; DFR, dihydroflavonol reductase; LDOX, leucoanthocyanidin dioxygenase; TT19, glutathione-S-transferase; HCT, hydroxycinnamoyltransferase; C3H, coumarate-3-hydroxylase; F5H, ferulate-5-hydroxylase; OMT, O-methyltransferase; SNG1, sinapoylglucose:malate sinapoyltransferase; CCOMT, caffeoyl-CoA O-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamoyl alcohol dehydrogenase. Adapted from Gachon *et al.* (2005) and http://www.arabidopsis.org/biocyc/.

To find the cause of reduced seed production we prepared transverse sections of flower buds at different developmental stages. Microscopy revealed that whilst pollen maturation and tapetum disintegration appeared normal in wild-type, anther dehiscence was delayed in the *35S-AtDOF4;2* lines (Figs. 2d-2g). This meant that most of the pollen grains were only released when the stigma was already above the anthers explaining the dramatic reduction in the number of produced seeds as well as the delayed silique formation.

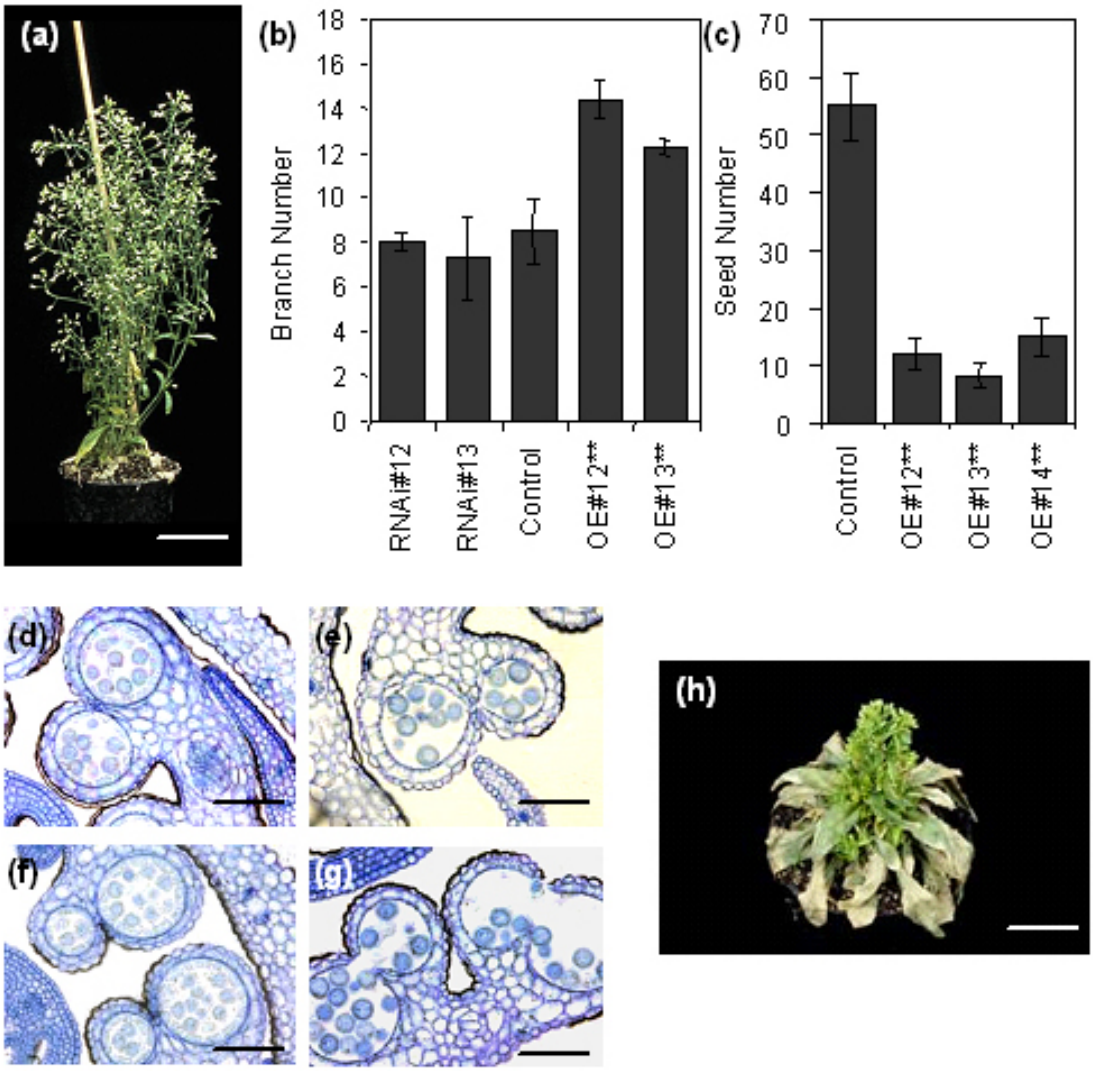

Figure 2. Phenotype of plants with modified *AtDOF4;2* expression. (a) Eight-week-old *35S-AtDOF4;2* transgenic plant (bar=2cm). (b) Number of branches emerging from rosette leaf axils in control (empty vector control), *35S-AtDOF4;2* (lines OE#12 and OE#13) and *RNAi-AtDOF4;2* (lines RNAi#12 and RNAi#13) plants. Data are means ± SD (n=8-12). (c) Seed number per silique in control (empty vector control) and *35S-AtDOF4;2* (lines OE#12, OE#13 and OE#14) plants. Data are means ± SD (n=12-15). (d) Cross section of the *35S-AtDOF4;2* anther at developmental stage 10/11. (e) Cross section of the *35S-AtDOF4;2* anther at developmental stage 13. (f) Cross section of the wild-type anther at developmental stage 10/11. (g) Cross section of the wild-type anther at developmental stage 13. Bars in d – g = 50µm. (h) Eight-week-old *35S-AtDOF4;2* transgenic plant after two weeks of growth in chilling conditions (bar=1.4cm). *Asterisks* (*) indicate values that are significantly different from the control plants (Student´s t-test; $p<0.05$).

4.3.2 Changes in flavonoid content

As some mutants with impaired dehiscence such as *coi1-16* (Ellis and Turner, 2002) can be rescued by low temperature, *35S-AtDOF4;2* plants were transferred to low temperature (16°C) under normal light intensity for 14 days. Surprisingly, leaves of *35S-AtDOF4;2* plants started to loose chlorophyll after 4-5 days and became senescent after 10-14 days (Figure 2h). A further experiment at 4°C confirmed this phenotype and showed it to be light dependent but revealed no differences for RNAi plants (data not shown). This phenotype seems most likely to be due to an increased sensitivity to light at low temperature. Increased sensitivity to light may reflect changes in photoprotective substances such as sinapate esters or flavonols (Havaux and Kloppstech, 2001). Flavonol measurements using HPLC/UV revealed no changes under normal growth conditions but when transferred to 4°C or high light *35S-AtDOF4;2* and *RNAi-AtDOF4;2* plants accumulated respectively less and more glycosylated flavonols (Table 1). Although HPLC/UV analysis allowed the detection of the relative changes for flavonol glycosides, and the quercetin and kaempferol glycosides and sinapic acid esters present in each peak were identified by LC/ESI/MS^n (Stobiecki *et al.,* 2006), it was not possible to assign changes to individual compounds (Table 1). Moreover, in extracts from plants grown in 4°C small amounts of quercetin 3-O-rhamnosyl-glucosyl-glucoside 7-O-rhamnoside and myricetin 3-O-rhamnosyl-glucoside 7-O-rhamnoside were detected by LC/ESI/MS^n but not HPLC/UV analysis (data not shown). Anthocyanin concentration was also lower in *35S-AtDOF4;2* and higher in *RNAi-AtDOF4;2* lines comparing to the wild-type plants (Table 2). Using quantitative RTPCR (Q-RTPCR) we measured the expression of genes involved in flavonol (early flavonoid biosynthetic genes) and anthocyanin (late flavonoid biosynthetic genes) biosynthesis and regulation. There were no changes in gene expression under standard growth conditions (data not shown), however upon cold stress the expression of *FLAVONOL SYNTHASE* (*FLS*), *DIHYDROFLAVONOL REDUCTASE* (*DFR*), *ANTHOCYANIDIN SYNTHASE* (*LDOX*) and *GLUTATHIONE TRANSFERASE* (*TT19*) were respectively lower and higher in *35S-AtDOF4;2* and *RNAi-AtDOF4;2* plants (Table 3), consistent with changed flavonol and anthocyanin content. In addition, *4-COUMARATE-COA-LIGASE (4CL3)* and *CHALCONE ISOMERASE* (*CHI*) expression was also reduced in *35S-AtDOF4;2* lines. Other genes of phenylpropanoid metabolism, namely *PAP1, PAL1, PAL2, PAL4, C3H, C4H, CCOMT1, CCOMT2,* and *F5H,* remained largely unchanged in the transgenic lines (data not shown).

Table 1. Determination of flavonols and sinapate esters in leaves of transgenic plants with altered *AtDOF4;2* expression. Metabolites were determined using HPLC/UV light detection in *35S-AtDOF4;2* (lines OE#12, OE#13 and OE#14), *RNAi-AtDOF4;2* (lines RNAi#12, RNAi#13) and empty-vector control plants (Control). Data are means ± SD (n=3). Samples were replicated with individual plants in one or two experiments. All samples were leaf samples taken from plants prior to bolting. Bold font and shading indicate values that are significantly different (Student´s t-test, $p<0.05$) in comparison to respective controls. Concentrations are given as the area of the flavonol peak/area of the internal standard (genistein/gFW^{-1}). A and 1, (retention time, RT ~33.4min) kaempferol 3-O-rhamnoside-7-O-rhamnoside and sinapoyl malate; B and 2, (RT~30.6min) isorhamnetin 3-O-glucoside 7-O-rhamnoside (detected in very low amounts) and kaempferol 3-O-glucoside-7O-rhamnoside; C and 3, (RT~28.9min) kaempferol 3-O-glucosyl-glucoside-7-O-rhamnoside and quercetin 3-O-glucosyl-7-O-rhamnoside; D and 4, (RT~26.1min) quercetin 3-O-glucosyl-glucoside-7-O-rhamnoside and kaempferol 3-O-rhamnosyl-glucoside-7-O-rhamnoside; E and 5, (RT~25.0min) quercetin 3-O-glucosyl-rhamnoside-7-O-rhamnoside; F and 5, (RT~22.5min) sinapoyl glucose and kaempferol 3-O-rhamnosyl-glucosyl-glucoside-7-O-rhamnoside. In Experiment 2, peak 5 was not fully resolved. The absolute values of metabolites in experiments 1 and 2 are different, which may be due to both, technical and/or biological variability.

Experiment1		**A**	**B**	**C**	**D**	**E**	**F**	**Sum**
Standard conditions Mean± SD	Control	1.07± 0.31	0.40± 0.12	0.57± 0.16	1.58± 0.53	0.04± 0.03	0.21± 0.17	3.87± 1.06
	RNAi#12	0.85± 0.11	0.29± 0.05	0.54 0.08±	1.32± 0.20	0.01± 0.01	0.24± 0.21	3.25± 0.4
	RNAi#13	1.03± 0.30	0.41± 0.18	0.55± 0.10	1.57± 0.48	0.02± 0.02	0.12± 0.07	3.7± 0.99
	OE#12	0.94± 0.27	0.56± 0.34	0.65± 0.19	1.71± 0.50	0.14± 0.16	0.28± 0.09	4.28± 0.95
	OE#13	0.99± 0.17	0.48± 0.18	0.74± 0.15	1.53± 0.17	0.08± 0.05	0.50± 0.30	4.32± 0.53
Cold Mean± SD	Control	3.89± 0.76	13.83± 1.72	8.91± 1.44	10.88± 0.92	2.28± 0.17	0.51± 0.18	40.3± 4.62
	RNAi#12	4.82± 0.53	21.44± 4.92	**18.88± 5.18**	16.08± 3.44	**4.82± 0.98**	0.91± 0.62	**66.95± 15.66**
	RNAi#13	4.16± 0.62	16.22± 0.25	**11.52± 0.88**	**12.55± 0.06**	**3.23± 0.37**	0.35 0.12	**48.03± 2.21**
	OE#12	**1.87± 0.22**	**5.78± 1.06**	**5.68± 0.49**	**6.54± 0.66**	**1.83± 0.11**	0.79± 0.25	**22.49± 2.54**
	OE#13	2.24± 0.43	**6.89± 2.80**	7.50± 0.43	**6.99± 1.47**	2.33± 0.12	0.63± 0.16	**26.58± 4.85**

Experiment2		**1**	**2**	**3**	**4**	**5**	**Sum**
Standard conditions Mean± SD	Control	1.18±0.12	0.45±0.08	1.10±0.13	2.21±0.21	0.38±0.06	5.32±0.48
	RNAi#12	1.32±0.23	0.51±0.07	1.26±0.18	2.63±0.46	0.60±0.22	6.32±1.16
	OE#14	1.19±0.15	0.62±0.08	1.11±0.14	2.65±0.33	0.54±0.07	6.11±0.76

Cold	Mean± SD	Control	2.45±0.16	4.41±0.28	2.09±0.16	5.24±0.07	0.61±0.04	14.8±0.71
		RNAi#12	2.33±0.03	**5.81±0.42**	2.58±0.32	**5.58±0.08**	**0.88±0.05**	**17.18±0.03**
		OE#14	**1.40±0.17**	**2.81±0.39**	2.16±0.28	**4.02±0.44**	**1.03±0.13**	**11.42±1.36**
High Light	Mean± SD	Control	3.14±0.06	4.51±0.20	4.36±0.07	6.19±0.55	2.23±0.27	20.43±0.61
		RNAi#12	**3.80±0.12**	5.69±0.74	**5.22±0.24**	7.41±0.41	2.61±0.38	**24.73±0.42**
		OE#14	**2.04±0.25**	**1.59±0.11**	3.95±0.22	5.41±0.54	2.56±0.17	**17.38±1.49**

Table 2. Determination of anthocyanins in leaves of transgenic plants with altered *AtDOF4;2* expression. Anthocyanins were determined spectrophotometrically in *35S-AtDOF4;2* (lines OE#12, OE#13, OE#14), *RNAi-AtDOF4;2* (lines RNAi#12, RNAi#13) and empty-vector control plants (Control). Data are means ± SD (n=6). Samples were replicated with individual plants in one experiment. All samples were leaf samples taken from plants prior to bolting. Bold font and shading indicate values that are significantly different (Student´s t-test, $p<0.05$) in comparison to respective controls. ND, not determined.

		Control	**RNAi#12**	**RNAi#13**	**OE#12**	**OE#13**	**OE#14**
Standard conditions	Mean±	0.64±	0.64±	0.70±	0.69±	0.64±	ND
	SD	0.06	0.08	0.15	0.12	0.16	ND
Cold	Mean±	1.00±	**1.77±**	**1.88±**	**0.71±**	**0.67±**	ND
	SD	0.27	**0.66**	**0.29**	**0.17**	**0.08**	ND
High light	Mean±	1.56±	**2.13±**	**2.57±**	ND	ND	1.40
	SD	0.15	**0.45±**	**0.82**	ND	ND	0.40

Table 3. Expression levels of genes involved in flavonoid biosynthesis in leaves of transgenic plants with altered *AtDOF4;2* expression.Transcript levels were measured using Q-RTPCR in *35S-AtDOF4;2* (lines OE#12, OE#14), *RNAi-AtDOF4;2* (lines RNAi#12, RNAi#13) and empty-vector control plants (Control). Data are means ± SD (n=3-4); samples were from one independent experiment replicated with individual plants. All samples were leaf samples taken from plants prior to bolting and grown at 4°C. Bold font and shading indicate values that are significantly different (Student´s t-test, $p<0.05$) in comparison to respective controls. ΔCt = Ct (gene) – Ct ((UBQ10+ACT2)/2); ΔΔCt= ΔCt (Control) – ΔCt (Transgenic); Change=$2^{\Delta\Delta Ct}$. ND, not determined.

Gene	Mean ΔCt ± SD					Change vs. Control				T-test			
	Control	RNAi#12	RNAi#13	OE#12	OE#14	RNAi#12	RNAi#13	OE#12	OE#14	RNAi#12	RNAi#13	OE#12	OE#14
DOF4;2	9.98± 0.12	**12.65± 1.1**	**13.3± 0.8**	**1.28± 0.25**	**1.33± 0.21**	**0.16**	**0.10**	**416**	**400**	**0.05**	**0.08**	**0.00**	**0.00**
4CL3	4.39± 0.45	4.37± 0.07	4.28± 0.17	**6.07± 0.84**	**5.51± 0.29**	1.01	1.08	**0.31**	**0.46**	0.96	0.77	**0.02**	**0.01**
CHS	1.68±0.7 0	1.07±0.1 4	1.07±0.2 6	1.73±0.6 8	1.20±0.5 2	1.52	1.53	0.97	1.4	0.31	0.32	0.92	0.37
CHI	2.03± 0.69	1.69± 0.03	1.61± 0.26	**3.28± 0.51**	3.03± 0.30	1.26	1.33	**0.42**	0.5	0.55	0.47	**0.03**	0.07
F3'H	6.46± 0.30	**5.28± 0.24**	**5.69± 0.46**	7.7± 1.11	7.51± 0.94	**2.26**	**1.7**	0.42	0.48	**0.01**	**0.03**	0.07	0.08
F3H	7.76± 0.39	7.33± 0.30	7.43± ND	8.57± 1.53	**6.93± 0.48**	1.35	1.26	0.57	**1.78**	0.25	ND	0.34	**0.05**
FLS	2.52± 0.26	**1.87± 0.20**	**1.83± 0.30**	**3.84± 0.76**	**3.5± 0.32**	**1.57**	**1.62**	**0.4**	**0.51**	**0.04**	**0.04**	**0.02**	**0.01**
DFR	6.33± 0.08	**4.9± 0.12**	**5.98± 0.21**	**9.00± 0.82**	**8.13± 0.97**	**2.7**	**1.27**	**0.16**	**0.29**	**0.00**	**0.03**	**0.00**	**0.03**
LDOX	5.92± 0.19	**4.9± 0.79**	5.59± 0.34	**7.56± 0.79**	**7.52± 0.71**	**2.03**	1.26	**0.32**	**0.33**	**0.05**	0.07	**0.01**	**0.01**
TT19	4.15± 0.32	**3.28± 0.45**	**3.32± 0.16**	**5.73± 0.85**	**5.16± 0.55**	**1.83**	**1.78**	**0.33**	**0.5**	**0.05**	**0.03**	**0.01**	**0.02**

4.3.3 Expression pattern

To further investigate the biological relevance of the changes in flavonoid content we measured *AtDOF4;2* steady-state transcript level in different plant organs using Q-RTPCR and by fusing approximately 1kb of *AtDOF4;2* 5' upstream promoter region to the *Escherichia coli* β–glucuronidase (GUS) reporter gene. Using Q-RTPCR we detected low transcript levels in leaves, root and stem, and high expression in flower buds at stages 10 and 11 (following the terminology of Bowmann, 1994; Figure 3a). In soil grown plants, weak GUS staining was observed in the main vein of leaves, whilst strong GUS activity was observed in the periderm of hypocotyls and primary roots after the transition to bolting, axillary meristems of the flower stalk, and the tapetum of anthers at developmental stage 10/11 (Figs. 3b-3e). Occasionally GUS staining was also observed in the testa of maturating seeds (Figs. 3f-3g). An identical GUS staining pattern was observed when a 2kb long promoter fragment was tested, arguing for 1kb containing all or most of the elements required for correct expression of the gene.

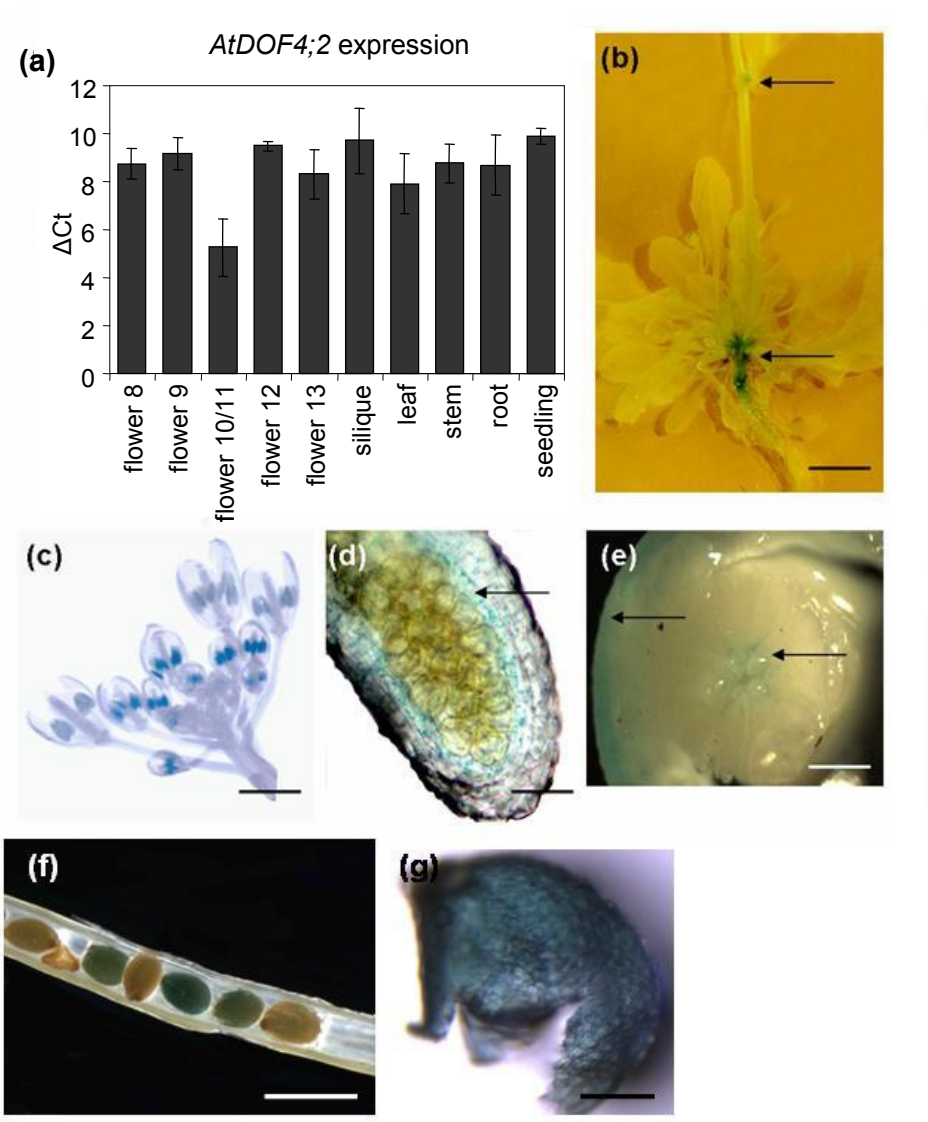

Figure 3. *AtDOF4;2* expression pattern.

(a) Q-RTPCR quantification of *AtDOF4;2* expression across different plant organs. Data are means ± SD (n=3-5). Flowers, siliques, leaves, stems, and roots were harvested from individual 6-7 week old wild-type plants. Agar grown seedlings were 10 days old. Highest expression (represented by lowest ΔCt value) was observed in flowers at stage 10/11. (b) – (e) Analysis of GUS activity in *promAtDOF4;2-GUS* plants. (b) Promoter-GUS line in flowering stage. Arrows indicate the hypocotyl and the axillary meristem. Weak GUS staining is present in the leaf main vein and in stem (bar=2cm). (c) Inflorescence. Staining is observed in flower buds at stages 10 and 11 (bar=2.5mm). (d) Longitudinal cross section of an anther at the stage 10/11. Arrow indicates the tapetum (bar=25µm). (e) Hand cut cross section of hypocotyl from an

8 week-old plant. Arrows indicate periderm and primary xylem (bar=0.6mm). (f) Silique filled with maturating seeds (bar=1mm). (g) Testa of the maturating seed (bar=125 µm).

4.3.4 Tapetum specific over-expression of *AtDOF4;2* leads to the formation of infertile, collapsed and flavonoid-free pollen grains

Because the CaMV 35S promoter may not drive expression in the tapetum (Plegt and Bino, 1989), in addition to *35S-AtDOF4;2* and *RNAi-AtDOF4;2* lines we generated *AtDOF4;2* over-expressing plants using the strong tapetum-specific promoter TA29 (Koltunow *et al.*, 1990). No phenotypic differences were observed in weak or moderate *TA29-AtDOF4;2* lines, however, strong over-expressers produced fewer seeds and the strongest line produced no seeds. Light microscopy and staining for flavonoids with diphenylboric acid 2-aminoethyl ester (DPBA) revealed the presence of abnormal, collapsed pollen grains that fluoresced blue under UV light, indicating the absence of flavonols in the walls of these microspores (Figs. 4a-4h). These abnormal pollen grains did not germinate; however, they had viable cytoplasm when treated with Alexander stain. Aberrant pollen grains were not associated with premature tapetum degeneration as this was not visibly affected in *TA29-AtDOF4;2* anthers (data not shown). To characterize *TA29-AtDOF4;2* plants further we measured expression of flavonoid genes in *TA29-AtDOF4;2* closed flower buds. Whilst there were no consistent changes in the expression of early flavonoid genes, the expression of late flavonoid genes (*DFR, LDOX* and *TT19*) and the MYB transcription factor *PAP1* were all significantly increased (Table 4).

Table 4. Expression levels of genes involved in phenylpropanoid biosynthesis or its regulation in transgenic plants with altered *AtDOF4;2* expression. Transcript level was measured using Q-RTPCR in (a), *TA29-AtDOF4;2* (line TA29#9), and wild-type (Col-0) plants and (b), *35S-AtDOF4;2* (line OE#12) and empty-vector control plants. Data are means ± SD (n=4-6); samples were from two independent experiments replicated with individual plants. All samples were (a) flower buds at stages 8-11 (Bowmann, 1994), or (b) hypocotyl samples taken from 7-8 week old plants. Bold font and shading indicate values that are significantly different (Student´s t-test, $p< 0.05$) in comparison to respective controls. ΔCt = Ct (gene) – Ct ((UBQ10+ACT2)/2); ΔΔCt= ΔCt (Control) – ΔCt (Transgene); Change = $2^{\Delta\Delta Ct}$. ND, not determined.

	(a)				(b)			
	Mean ΔCt±SD		Change vs. Col-0	T-test	Mean ΔCt±SD		Change vs. Control	T-test
	Col-0	TA29#9	TA29#9		Control	OE#12	OE#12	
AtDOF4;2	10.46±0.60	**1.79±0.83**	**407.31**	**0.000**	ND	ND	ND	ND
PAL1	1.86±0.94	**0.26±0.86**	**3.03**	**0.005**	**2.89±0.07**	**1.05±0.82**	**3.61**	**0.018**
PAL2	2.43±0.58	**1.24±0.72**	**2.28**	**0.008**	**2.95±0.13**	**2.02±0.34**	**1.89**	**0.042**

PAL3	6.46±0.38	**5.69±0.34**	**1.71**	**0.038**	9.03±1.48	8.22±1.03	1.74	0.764
PAL4	2.98±0.18	**2.22±0.30**	**1.69**	**0.013**	3.57±0.27	2.94±0.56	1.54	0.078
C3H	3.73±1.08	3.11±0.56	1.54	0.207	2.62±0.67	2.26±1.13	1.29	0.532
C4H	0.59±0.49	**-0.33±0.70**	**1.89**	**0.010**	**3.96±0.74**	**2.20±1.10**	**3.39**	**0.024**
HCT	3.98±0.56	3.57±0.41	1.33	0.301	ND	ND	ND	ND
4CL1	2.66±0.08	2.31±0.56	1.27	0.351	0.02±1.44	-0.34±1.89	1.29	0.766
4CL2	4.86±0.32	5.26±0.43	0.76	0.236	2.22±1.14	2.10±1.45	1.09	0.898
4CL5	5.30±0.51	**4.26±0.33**	**2.06**	**0.001**	**3.16±0.49**	**1.58±0.31**	**2.99**	**0.005**
CCOMT1	0.51±0.50	**-0.60±0.24**	**2.16**	**0.012**	3.85±1.00	2.98±0.65	1.82	0.42
CCOMT2	8.71±0.85	**7.74±0.55**	**1.96**	**0.023**	4.44±0.66	4.87±0.38	0.74	0.217
CCOMT4	11.24±1.45	10.30±0.75	1.87	0.375	**11.39±0.89**	**7.93±0.98**	**10.97**	**0.004**
F5H	5.55±0.57	5.30±0.49	1.19	0.559	ND	ND	ND	ND
4CL3	3.40±0.39	**2.34±0.24**	**2.08**	**0.006**	ND	ND	ND	ND
CHS	2.35±0.87	1.70±1.07	1.57	0.214	ND	ND	ND	ND
CHI	2.43±0.23	2.67±0.87	0.85	0.467	ND	ND	ND	ND
FLS	1.69±0.35	1.37±0.56	1.25	0.200	ND	ND	ND	ND
DFR	10.77±0.71	**9.52±0.42**	**2.27**	**0.001**	ND	ND	ND	ND
LDOX	8.80±0.91	**7.31±0.88**	**2.81**	**0.007**	ND	ND	ND	ND
TT19	6.14±0.69	**4.70±0.64**	**2.71**	**0.001**	ND	ND	ND	ND
PAP1	8.83±1.01	**7.21±0.55**	**3.07**	**0.002**	ND	ND	ND	ND

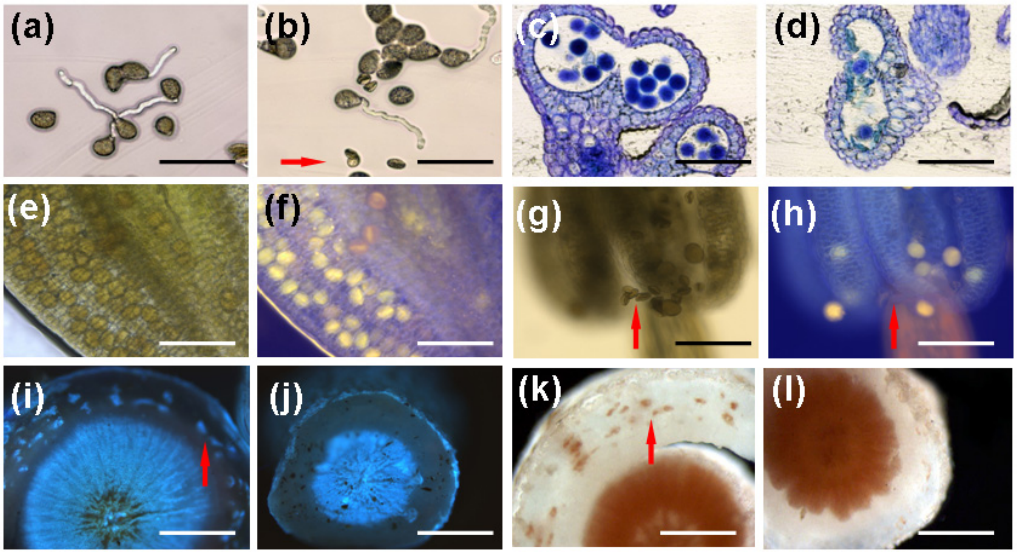

Figure 4. Phenotype of plants with modified *AtDOF4;2* expression.(a) Wild-type pollen grains on germination medium. (b) *TA29:AtDOF4;2* pollen grains on germination medium. Note the small, abnormal pollen grains that do not produce pollen tubes (arrow). (c) Section of wild-type anther prior to dehiscence. (d) Section of *TA29:AtDOF4;2* anther prior to dehiscence. (e, f) DPBA stained wild-type anthers observed under visible and UV light. Note bright yellow fluorescence of the pollen grains when viewed under UV light (f). (g, h) DPBA stained *TA29:AtDOF4;2* anthers observed under visible and UV light. Note the lack of

fluorescence in the arrow-indicated aberrant pollen grains. (i) Hand cut transverse section of a wild-type hypocotyl observed under UV light; the arrow indicates a phloem fibre. (j) Hand cut transverse section of a *35S-AtDOF4;2* hypocotyl observed under UV light. (k) Hand cut transverse section of a wild-type hypocotyl stained with phloroglucinol (l) Hand cut transverse section of a *35S-AtDOF4;2* hypocotyl stained with phloroglucinol. Bars in (a, b) = 35µm, in (c - h) = 50µm, in (i, j) = 0.8 mm, in (k, l)=0.6mm.

4.3.5 Tissue specific expression analysis of the wider phenylpropanoid pathway

The tapetum produces sporopollenin, a lignin-related biopolymeric substance that is deposited onto pollen grains and has both protective and structural functions. It is composed mainly of long-chain fatty acids and of phenolic compounds, p-coumaric and ferulic acid, coupled by ester bonds (Piffanelli *et al.*, 1998; Scott *et al.*, 2004). High expression level of *AtDOF4;2* in tapetum, and the observed changes in flavonoid metabolism prompted us to investigate the expression of key genes in other areas of phenylpropanoid metabolism. Expression of genes encoding for the first two enzymes in phenylpropanoid metabolism, *PHENYLALANINE AMMONIA LYASE* (*PAL1-4*) and *CINNAMATE-4-HYDROXYLASE* (*C4H*) was significantly increased. Also increased were *CAFFEOYL-COA-METHYLTRANSFERASE* (*CCOMT1, CCOMT2*) and *4-COUMARATE-COA-LIGASE* (*4CL5*), whilst the expression of *COUMARATE-3-HYDROXYLASE* (*C3H*), *4-COUMARATE-COA-LIGASE* (*4CL1* and *4CL2*) and *HYDROXYCINNAMATE TRANSFERASE* (*HCT*) was not affected (Table 4).

Suberin is a biopolymeric substance chemically similar to sporopollenin deposited in the periderm of root (Nawrath, 2002). As *AtDOF4;2* is also highly expressed in the periderm we harvested hypocotyls of the flowering *35S-AtDOF4;2* plants and analyzed expression of the phenylpropanoid genes. Similar to *TA29-AtDOF4;2* flower buds expression of *PAL1, PAL2, C4H* and *4CL5* was higher in *35S-AtDOF4;2* hypocotyls. Expression of *CCOMT1* and *CCOMT2* was not affected but the transcript level of *CCOMT4* was elevated (Table 4). Additionally we examined lignin deposition in transgenic hypocotyls using phloroglucinol staining. In contrast to control plants lignin fibers of the over-expressers did not stain indicating changes in lignin content (Figs. 4i-4l).

4.3.6 Transcriptional regulation of *AtDOF4;2* expression

To investigate the transcriptional regulation of *AtDOF4;2* we measured its expression level using Q-RTPCR in leaves of plants subjected to various treatments known to influence phenylpropanoid metabolism. We detected no changes in response to high light, cold or upon wounding. *AtDOF4;2* expression was also not effected by nitrate starvation (Scheible *et al.*,

2004). *AtDOF4;2* was moderately decreased (2-fold) by infection with the fungal pathogen *Altenaria brassicicola* and jasmonate treatment both measured after 6h (McGrath *et al.*, 2005).

4.4 Discussion

4.4.1 Bushy phenotype of *35S-AtDOF4;2* plants.

During a wider screen of plants over-expressing DOF transcription factors we found that *AtDOF4;2* over-expression caused a bushy phenotype due to released axillary bud growth. Similar phenotypes have been associated with changes in auxin transport or perception. Increased branching can be associated with both decreased and increased basipetal auxin transport as illustrated by the *tir-3* and *max1-4* mutants (Bennett *et al.*, 2006; Ruegger *et al.*, 1997). In the *axr1* mutant, increased branching is caused by insensitivity to acropetally supplied auxin (Lincoln *et al.*, 1990). Increased branching of *35S-AtDOF4;2* plants was associated with reduced ^{3}H-IAA transport in the stem compared to wild-type, whilst excised bud assays showed no difference in sensitivity to acropetally supplied auxin (Supplemental Figure 1a-b). This indicates that the *35S-AtDOF4;2* phenotype is caused by a defect in auxin transport, however, as we measured no changes in *RNAi-AtDOF4;2* plants it is likely to be a secondary effect of AtDOF4;2 function. For example it has been previously shown that developing fruits inhibit lateral branching (Hense *et al.,* 1994), therefore, the reduced seed production in *35S-AtDOF4;2* plants resulting from delayed anther dehiscence may be indirectly responsible for the observed bushy phenotype. Our further analyses revealed AtDOF4;2 influenced phenylpropanoid metabolism. Such a link has been previously described for a *MYB26* loss-of-function mutant which is characterized by changes in phenylpropanoid metabolism (i.e. lignin deposition), lack of dehiscence and increased branching (Steiner-Lange *et al.*, 2003).

4.4.2 *AtDOF4;2* negatively affects flavonoid biosynthesis

In contrast, during vegetative growth plants over-expressing *AtDOF4;2* are indistinguishable from wild-type plants. However, when grown at low temperatures (4°C) in the light, but not in the dark, leaves of *35S-AtDOF4;2* plants loose chlorophyll and eventually die. These conditions are known to cause photooxidative and photoinhibitory stress by damaging chloroplasts which suggests *35S-AtDOF4;2* plants have a reduced capacity to photoacclimate. Photoacclimation during long-term exposure to chilling stress in the light results from the accumulation of flavonoids, particularly light absorbing flavonols that possess UV/blue light trapping properties and decrease chlorophyll excitation by blue light (Havaux and Kloppstech, 2001). For example,

the *transparent testa5* (*tt5*) mutant in *CHALCONE ISOMERASE* (*CHI)* completely lacks flavonols and suffers from extensive photooxidative and photoinhibitory damage to the chloroplast when grown under chilling conditions (Havaux & Kloppstech, 2001). Photoprotective roles have also been demonstrated for anthocyanins which protect against green light under chilling conditions (Havaux & Kloppstech, 2001). We therefore decided to profile flavonols and anthocyanins in wild-type, *35S-AtDOF4;2* and *RNAi-AtDOF4;2* plants grown under normal, cold or high-light conditions. LC/UV measurements of glycosylated flavonols and spectrophotometric determination of anthocyanins revealed no changes under normal growth conditions but both changed significantly under cold and high-light conditions. Consistent with changes in flavonoid metabolism being responsible for the *35S-AtDOF4;2* phenotype observed at low temperature, flavonol and anthocyanin concentrations were respectively decreased and increased in *35S-AtDOF4;2* and *RNAi-AtDOF4;2* plants. As low-temperature causes massive metabolic changes (Cook *et al.*, 2004; Kaplan *et al.*, 2004) it was possible that changes in flavonoid metabolism were associated with wider metabolic differences. However, GC/MS metabolite profiling of the same samples revealed no such reciprocal differences for any of the routinely detected metabolites, including the phenylpropanoid precursors shikimate and phenylalanine, which together argue for specific changes in flavonoid metabolism. These data indicate that AtDOF4;2 may, directly or indirectly, negatively regulate flavonoid content specifically under stress conditions (Figure 5). Other negative regulators of phenylpropanoid metabolism including MYB4 and its ortholog from *Antirrhinum majus* AmMYB308 have been described previously, yet these also cause changes under normal growth conditions (Hemm *et al.*, 2001; Jin *et al.*, 2000; Tamagnone *et al.*, 1998). It was shown that under UV stress or upon wounding expression of MYB4 is reduced and as a result biosynthesis of sinapate esters is induced. To investigate whether AtDOF4;2 has a similar mode of action we checked its expression under different environmental conditions known to influence flavonoid content. Unfortunately, *AtDOF4;2* is not represented on the Affymetrix GeneChip ATH1 array, and is below the sensitivity for most experiments using the AtGenome array on which it was represented. Therefore, we mined publicly available Q-RTPCR expression data to extract expression information for *AtDOF4;2*. *AtDOF4;2* was reduced in response to methyl jasmonate treatment and infection with *Alternaria brassicicola*, conditions which stimulate anthocyanin biosynthesis (McGrath *et al.*, 2005). However, neither cold nor high light influenced its expression. It is possible that AtDOF4;2 is specific for the biotic stimuli but more likely its activity is modulated on the level of protein or protein-protein interaction. The need of additional, stress induced factors would explain why

35S- and RNAi-mediated changes in *AtDOF4;2* expression are not sufficient to influence flavonoid levels under normal growth conditions.

Promoter-GUS analysis and Q-RTPCR showed that *AtDOF4;2* is highly expressed in the tapetum specifically at flower stages 10 and 11. The tapetum is responsible for supplying developing pollen with metabolic resources (Piffanelli *et al.*, 1998; Scott *et al.*, 2004). Among compounds provided by the tapetum are flavonoids, which are incorporated into the pollen coat where they influence pollen pigmentation and structure, playing important roles in protection against UV radiation and pathogen attack (Piffanelli *et al.*, 1998). Analysis of pollen grains produced by plants expressing *AtDOF4;2* under a strong tapetum-specific promoter (TA29) revealed them to be completely devoid of flavonols. As we observed no differences in tapetum development this was most likely related to a negative effect of AtDOF4;2 on flavonoid biosynthesis. Our *promoter:GUS* studies also found coincident changes in *AtDOF4;2* expression and phenypropanoid biosynthesis in other tissues. In the periderm, formed during secondary thickening of the main root, high *AtDOF4;2* expression was coincident with a lack of flavonoids in dark grown roots (Hemm *et al.*, 2004).

Several studies have shown that changes in flavonoid accumulation are regulated by transcriptional changes in biosynthetic enzymes. To investigate if the changes we observed were also manifest at the transcriptional level we investigated selected biosynthetic genes using Q-RTPCR on *35S-AtDOF4;2* and *RNAi-AtDOF4;2* plants subjected to cold and light. We detected opposite expression changes of *FLAVONOL SYNTHASE* (*FLS*), *DIHYDROFLAVONOL REDUCTASE* (*DFR*), *ANTHOCYANIDIN SYNTHASE* (*LDOX*) and *GLUTATHIONE TRANSFERASE* (*TT19*) which were likely responsible for the observed changes in flavonoids (Figure 5). Interestingly, these changes were not mirrored by consistent changes in the expression of the MYB transcription factor *PAP1*, which has been shown to regulate expression of *DFR, LDOX* and *TT19* genes (Tohge *et al.*, 2005). Whether AtDOF4;2 directly or indirectly regulates these genes is unknown. All of these genes harbor multiple potential DOF binding sites in their promoter regions; however, as these sites are relatively abundant genome-wide direct binding evidence would need to be obtained. As DOF TFs have been shown to have activator and repressor activity (Yanagisawa, 2004), direct negative regulation of these genes is possible. For example, maize DOF1 and DOF2 were shown to have opposite effects on C4-type *PHOSPHOENOLPYRUVATE CARBOXYLASE* gene expression (Yanagisawa & Sheen, 1998). Another possibility, particularly considering the described MYB regulators of flavonoid biosynthesis, would be the previously demonstrated interactions between MYB and DOF TFs (Diaz *et al.*, 2002; Isabel-LaMoneda *et al.*, 2003).

4.4.3 Wider changes in phenylpropanoid metabolism caused by AtDOF4;2

Changes in flavonoid biosynthesis may have wider consequences for phenylpropanoid metabolism by re-directing the pool of the common precursor 4-coumarate into lignin or hydroxycinnamic acid production. The tapetum and periderm, both characterized by strong *AtDOF4;2* expression, produce hydroxycinnamic acids, mainly coumaric acid and ferulic acid, which together with fatty acids are used to produce the biopolymers sporopollenin and suberin (Franke *et al.*, 2005; Piffanelli *et al.*, 1998; Wiermann & Gubatz, 1992). Suberin is a structural polymer that protects the roots against water loss and pathogen attack whilst sporopollenin is the major constituent of the outer cell wall of pollen grains having both protective as well as structural properties. The pollen of the *ms2* and *dex-1* mutants, defective in sporopollenin formation, is collapsed and consequently sterile indicating sporopollenin is necessary for the development of viable pollen grains (Aarts *et al.*, 1997; Paxson-Sowders *et al.*, 2001). *TA29-AtDOF4;2* plants produce similar, collapsed, infertile pollen grains. This phenotype cannot be explained simply by the observed absence of pollen coat flavonols as Arabidopsis transparent *tt4* mutant, that is completely devoid of flavonoids, produces normal pollen grains (Shirley *et al.*, 1995). Both increased and decreased expression of specific hydroxycinnamic acid biosynthetic genes has been previously shown to be associated with aberrant pollen formation (Preston *et al.,* 2004), suggesting that sporopollenin formation and/or function may be sensitive to changes in the pools of specific hydroxycinnamic acids. In *AtDOF4;2* over-expressing lines specific hydroxycinnamic acid biosynthetic genes are consistently increased in both flower buds and hypocotyls, which may alter the pool composition of hydroxycinnamic acids. Alternatively, it is possible that the down-regulation of flavonoid biosynthesis shifts the pool of coumarate into hydroxycinnamate production; particularly as such interaction was described previously for Arabidopsis roots where light exposure stimulates flavonoid accumulation and a reduction in ferulic acid (Hemm *et al*., 2004). Furthermore, these mechanisms do not need to be exclusive and the observed expression changes throughout phenylpropanoid metabolism could be interlinked. Regardless of the exact mechanism of change, it is more likely that defective pollen grains in *TA29-AtDOF4;2* plants are caused by altered composition of the hydroxycinnamic acid pool leading to changes in the composition and structural properties of sporopollenin. These expression changes were organ-/tissue-specific as they were not detected in leaves of *35S-AtDOF4;2* plants. This may indicate that AtDOF4;2 interacts with other proteins in the regulation of phenylpropanoid metabolism and this interaction occurs only in certain conditions or tissues. These data highlight the specificity of tissue and stimulus dependent changes in phenylpropanoid metabolism.

Whether hydroxycinnamic acid synthesis is the primary target of AtDOF4;2 has to be investigated further. Intriguing is a possible negative and positive function of AtDOF4;2 in transcriptional control of flavonoid and hydroxycinnamic acid production, respectively (Figure 5). Such positive and negative regulation of multiple target promoters was previously shown for barley DOF transcription factor PBF that activates transcription from the B-hordein gene promoter but represses transcription from the cathepsin B-like protease gene promoter (Mena *et al*., 2002).

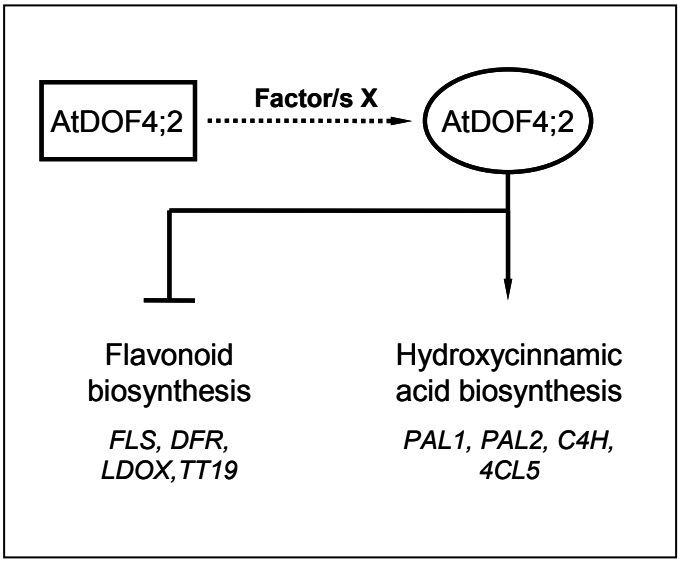

Figure 5. Proposed model of AtDOF4;2 function in plants. We speculate that the activity of transcription factor AtDOF4;2 is modulated at the protein level, e.g. through protein-protein interaction, by unknown stress- or tissue-specific factor/s X. Upon activation AtDOF4;2 affects phenylpropanoid metabolism in a tissue- and stimulus-dependent manner. It negatively influences flavonoid biosynthesis by repressing expression of genes such as *FLS, DFR, LDOX* and *TT19,* but stimulates expression of genes such as *PAL1-2, C4H* and *4CL5* responsible for the production of hydroxycinnamic acids.

4.5 Materials and Methods

4.5.1 General methods

Standard molecular techniques were performed as described (Sambrook *et al*., 2001). Oligonucleotides were obtained from MWG (Ebersberg, Germany). DNA sequencing was performed by AGOWA (Berlin, Germany). Unless otherwise indicated, other chemicals were purchased from Roche (Mannheim, Germany), Merck (Darmstadt, Germany), or Sigma (Deisenhofen, Germany). For sequence analyses the tools provided by the National Center for

Biotechnology Information (http://www.ncbi.nlm.nih.gov/), MIPS (http://mips.gsf.de/), The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/), and the Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de) were used.

4.5.2 Plant material

Arabidopsis seeds were sown in soil (Einheitserde GS90; Gebrüder Patzer, Sinntal-Jossa, Germany) and grown in a growth chamber with a 16-h day length provided by fluorescent light at 80 or 120 µmol m^{-2} s^{-1} and a day/night temperature of 20/16°C and relative humidity of 60/75%. *Agrobacterium tumefaciens* strain GV3101 (pMP90) was used to transform *Arabidopsis thaliana* (L.) Heynh. cv. C24 (*35S-AtDOF4;2, RNAi-AtDOF4;2, promAtDOF4;2-GUS*) or Col-0 (*TA29-AtDOF4;2*) (Clough, 2005). For cold experiments plants were transferred to a 4 °C growth chamber with a 16-h photoperiod at 90 µmol m^{-2} s^{-1} and for chilling experiments to a 16°C growth chamber with a 16-h photoperiod at 120 µmol m^{-2} s^{-1}. For high light treatment a growth chamber with a 16h photoperiod at 600 µmol m^{-2} s^{-1} was used. If not indicated differently measurements were performed on fully developed rosettes harvested from randomized 5- to 6-week-old plants prior to bolting.

4.5.3 Constructs

35S-AtDOF4;2: Polymerase chain reaction (PCR) was used to amplify the *AtDOF4;2* coding region using Arabidopsis C24 leaf cDNA as template. Primer sequences were as follows: *DOF4;2*-fwd., 5´-GTTTAAAC ATGAATAATTTGAATGTTTTTA-3´ (added *Pme*I restriction site underlined); *DOF4;2*-rev., 5´-TTAATTAATTATGATTCATATTCAAATTGC-3´ (added *Pac*I restriction site underlined). The *AtDOF4;2* cDNA was inserted into pUni/V5-His-TOPO (Invitrogen, Karlsruhe, Germany) and, after sequence confirmation, cloned via the *Pme*I/*Pac*I sites into a modified pGreen0229 plant transformation vector (www.pgreen.ac.uk) containing a Cauliflower Mosaic Virus (CaMV) 35S promoter and *Pme*I/*Pac*I restriction sites (Skirycz *et al*., 2006). ***TA29-AtDOF4;2:*** Primer sequences were as follows: *DOF4;2*-fwd., 5´-GGATCCATGAATAATTTGAATGTTTTTACAAAT-3´ (added *Bam*HI restriction site underlined); *DOF4;2*-rev., 5´-GAATTCTTATGATTCATATTCAAATTGCAA-3´ (added *Eco*RI restriction site underlined). The *AtDOF4;2* cDNA was inserted into vector TOPO-TA (Invitrogen) and, after sequence confirmation, cloned via the *Bam*HI and *Eco*RI sites into a pGreen vector containing the TA29 promoter cloned from TA29 pSVB30 vector kindly provided by Andreas Perlick (Universität Bielefeld, Germany). ***RNAi-AtDOF4;2:*** A 3'-UTR *AtDOF4;2* fragment, which does not share similarity with the UTRs of the *AtDOF4;2* homologous genes *AtDOF4;3, AtDOF4;4,*

AtDOF4;5, was PCR amplified from a leaf cDNA library using the oligonucleotides RNAi-fwd 5´-CACCTGGAAGGGATTTTTAGGCACTGAGATGAAC-3´ and RNAi-rev 5´-AAGATAGAAAACAAGTAATACAATTAAAATTG -3'. The PCR product was cloned into pENTR/D-TOPO of the Gateway System (Invitrogen). After sequencing, the *AtDOF4;2* fragment was transferred to the destination vector pJawohl8-RNAi (kindly provided by Dr. Imre Somssich, MPI for Plant Breeding, Cologne). ***Promoter-GUS fusion*****:** A ~1-kb 5´ genomic fragment upstream of the ATG start codon was amplified by PCR using primers *DOF4:2*GUS-fwd (5´-GTCGACCTCAAAACAAATCTCTAGCTGCTT-3´) and *DOF4:2*GUS-rev (5´-TCTAGAATTCTCTCGAGAGGTTGTTTATTTT-3´) from Arabidopsis C24 genomic DNA. The promoter fragment was inserted into plasmid pCR-Blunt II-TOPO (Invitrogen) and verified by sequencing. Subsequently, the promoter was fused to the *E. coli* β-glucuronidase (GUS) reporter gene in pGPTV-Kan (Becker *et al.*, 1992), previously cut with *Sal*I and *Xba*I, resulting in plasmid *promAtDOF4;2-GUS*.

4.5.4 GUS assays

β-Glucuronidase activity was determined histochemically as described (Plesch *et al.*, 2001) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as substrate (Jefferson *et al.*, 1987).

4.5.5 Anatomical analysis using light microscopy

Tissues were fixed in FAA solution (5% (v/v) acetic acid, 50% (v/v) ethanol, 3.7% formaldehyde (v/v)) and left overnight at room temperature. Dehydration was carried out through an ethanol series (50%, 70%, 90%, 95%, and 100%) at room temperature with 2h - 4h per step. After dehydration the tissue was infiltrated for 2h in a solution containing 50% (v/v) ethanol and 50% (v/v) Technovit 7100 (Heraeus-Kulzer, Wehrheim, Germany). The samples were then incubated overnight in a solution of 1% Hardener I in 100% Technovit 7100. Polymerization was carried out by adding Hardener II. Sections of 4µm thickness were cut using histo-knives with a microtome and stained with 0.1% (w/v) toluidine blue. Cross sections were analyzed using an Olympus BX 41 System Microscope.

4.5.6 Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) as described in the manual. RNA (2 µg) was digested with DNase (Sigma) and reverse transcribed with Superscript III reverse transcriptase (Invitrogen) in a reaction volume of 20 µl to generate first-strand cDNA.

Quantitative RT-PCR was performed in 96 well-plates with 1 µl of a 1:4 dilution of the first-strand cDNA reaction and SYBR Green reagent (Applied Biosystems Applera, Darmstadt, Germany), in a 10-µl volume, on a GeneAmp 7300 Sequence Detection System (Applied Biosystems). Primer sequences are given in Table S1 in the supplementary material. Data were normalized to *ACTIN2* and *UBIQUITIN10*; ΔCt = Ct (gene) – Ct ((UBQ10+ACT2)/2). Ct refers to the number of cycles at which SYBR Green fluorescence in a PCR reaches an arbitrary value during the exponential phase of DNA amplification, set at 0.2 in all experiments.

4.5.7 Anthocyanin measurements

Anthocyanin extraction and determination was performed as described in Mehrtens *et al.* (2005).

4.5.8 Isolation of flavonol compounds

Plant material was frozen in liquid nitrogen and powdered in a Retsch mill (Retsch, Hahn, Germany). For the extraction of flavonoid glycosides plant material (~1g fresh weight per sample) was homogenized in 10 ml of 80% (v/v) methanol, and the suspensions were placed in an ultrasonic bath for 30 min. Genistein (Extrasynthese, Genay, France) was added as an internal standard to each analyzed sample prior to the extraction procedure, the final concentration of the standard in the samples injected into the LC column being about 0.05 µg/µl. The extracts were filtered through a Büchner funnel and concentrated under reduced pressure on a Rotovapour at a bath temperature of 40 °C. The dried sample was dissolved in 500 µl of 50% methanol and transferred to the LC vial, the flask was washed with the next portion of 500 µl of 50% methanol and both volumes were combined in the vial.

4.5.9 Liquid chromatography coupled to UV light or mass spectrometric (LC/ESI/MS^n) detection

LC analyses of plant extracts were performed with two LC systems combined with two different types of detectors. LC/UV analyses were performed on an Merck Hitachi HPLC system: pump Model L-7100, diode array detector Model L-7450, automatic injector Model L-7200 and degasser Model 7614 (Darmstadt, Germany). The second instrument used was an Agilent 1100 HPLC pump (Palo Alto, CA, USA) combined with an ion trap (IT) mass spectrometer, model Esquire 3000 (Bruker Daltonics, Bremen, Germany), with electrospray ionization (ESI) sources. IT source parameters were as follows: ESI source voltage of 4 kV, nebulization with nitrogen at 30 psi, dry gas flow of 9.0 l/min at temperature of 310 °C, skimmer 1 voltage 12.4 or 44.2 V,

collision energy set to 30%. The instrument operated under EsquireControl version 5.1, and data were analyzed using the Data Analysis version 3.1 package delivered by Bruker.

In both systems 5 µl samples were injected onto a Superspher 100 RP-18 column (250 x 2 mm; Merck), at a 0.2 ml/min flow rate. During liquid chromatography, the elution from the LC column was carried out using mixtures of two solvents: A (95% acetonitrile, 4.5% H_2O, 0.5% formic acid, v/v/v) and B (95% H_2O, 4.5% acetonitrile, 0.5% formic acid, v/v/v). The elution steps were as follows: 0–5 min isocratic at 10% A, 5–40 min linear gradient from 10 to 30% of A, 40–48 min linear gradient up to 100% of A, 48–60 min isocratic at 100% of A. Flavonol glycosides were identified by their MS spectra and/or by their retention times compared with the data obtained for standards (Stobiecki *et al*., 2006).

4.5.10 Analysis of pollen

Pollen grains from soil grown plants were examined using an Olympus BX 41 System Microscope. Pollen grains were mounted in a drop of Alexander's stain (Alexander, 1969) or DPBA solution (Ylstra *et al.*, 1996), under a cover-slip. Germination assays were performed as described in (Azarow *et al.,* 1990).

4.5.11 Phloroglucinol staining

Phloroglucinol staining of hand cross-sections of dark grown hypocotyls was carried out as follows: samples were placed in a 1% phloroglucinol-HCl solution for 10 min, mounted in 50% glycerol, 6 N HCl and observed immediately.

4.5.12 AGI code numbers

The AGI code numbers for the genes tested in this study are as follows: At3g18780 (*ACT2*); At4g05320 *(UBQ10)*; At4g21030 (*AtDOF4;2*); At1g65060 (*4CL3*); At5g13930 (*CHS*); At3g55120 (*CHI*); At3g51240 (*F3H*); At5g07990 (*F3'H*); At5g08640 (*FLS*); At5g42800 (*DFR*); At5g17220 (*TT19*); At4g22880 (*LDOX*); At1g56650 (*PAP1*); At2g37040 (*PAL1*); At3g53260 (*PAL2*); At5g04230 (*PAL3*); At3g10340 (*PAL4*); At2g30490 (*C4H*); At2g40890 (*C3H1*); At3g212304 (*4CL5*); At1g51680 (*4CL1)*; At3g21240 (*4CL2)*; At4g34050 *(CCOMT1)*; At4g26220 *(CCOMT2)*; At1g67980 *(CCOMT4)*; At5g48930 *(HCT)*; At4g36220 *(F5H1)*.

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5. DOF transcription factor OBP1 (AtDof3.4) is involved in cell cycle regulation

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Authors´ contributions

Bernd Mueller-Roeber conceived the idea for the study. The research was designed and planned by Bernd Mueller-Roeber and Aleksandra Skirycz. The experimental work was done by Aleksandra Skirycz. The research was supported through collaborations as follows: ploidy measurements were performed in collaboration with Amandine Radziejwoski and Lieven De Veylder; Wolfgang Busch and Jan Lohmann helped to establish chromatin immuno-precipitation experiments; Matthew Hannah analyzed Affymetrix expression data; Mirosław Kwaśniewski assisted in the elctrophoretic-mobility shift assays; Maria-Inès Zanor and Isabell Witt prepared the *35S-OBP1* construct. Joanna Czeszejko assisted as a student worker in the data collection; she was supervised by Aleksandra Skirycz.

5.1 Abstract

- Precise regulation of the cell cycle is of central significance for plant growth and development and our work has uncovered DOF transcription factor (TF) OBP1 that is involved in control of cell division upon developmental signaling.
- To identify biological processes regulated by OBP1 we performed expression profiling of *OBP1* inducible lines and identified significant over-representation of cell cycle related genes among up-regulated transcripts. Direct targets of OBP1, verified by chromatin immunoprecipitation and electrophoretic-mobility shift assays, include core cell cycle gene *CYCD3;3* and replication specific transcription factor *AtDOF2;3*. Consistent with our molecular data short term activation of OBP1 in cell cultures affects duration of the G1 phase and overall shortens cell cycle length whilst constitutive over-expression of *OBP1* in plants influences cell size and cell number leading to a dwarfish phenotype. Moreover data were obtained supporting the model in which AP2 TF ENHANCED SHOOT REGENERATION 2 regulates expression of *OBP1*.
- Finally to establish the biological context in which OBP1 affects cell division we looked at the overlap in the expression patterns of *OBP1*, its target genes and *ESR2* and we gained evidence for OBP1 involvement in cell cycle activation in the root apex during seed germination and in cotyledon initiation during embryogenesis.

5.2 Introduction

Cell proliferation plays a prominent role in the growth and development of multicellular organisms. Plant cell division shares its basic mechanism with all eukaryotes. It consists of the replication (S) and mitotic (M) phases separated by two gap phases; G1 and G2. Additionally, some cells can skip mitosis, in a process called endoreduplication, leading to increased cell ploidy (i.e. chromosome content).

Progression of the cell cycle is tightly regulated and ensured by deploying transcriptional, post-transcriptional and post-translational control mechanisms (reviewed by Inze and deVeylder, 2006 and 2007). A central role is played by cyclin dependent kinases (CDKs) and their phosphorylated targets that trigger the onset of DNA replication and mitosis. Direct protein-protein interactions are also important, for example, as their name implies, CDKs only act in complexes with various cyclin proteins. In addition, the abundance of cell cycle components provides additional regulation. Targeted protein degradation provides an irreversible mechanism that pushes the cell cycle forward by targetting cyclins, E2F transcripton factors and Kip-related

proteins. Cell cycle progression is controlled at the transcriptional level by the E2F and R1R2R3 MYB TF families which regulate replication and mitosis, respectively.

The commitment of cells to the mitotic cycle is an essential control step. In plants, the activity of the cyclin D (CYCD)/retinoblastoma (RB) pathway is considered of primary significance for controlling this step. Binding of CYCD proteins to PSTAIRE-containing cyclin dependent kinase A (CDKA) allows it to phosphorylate the RB protein leading to its inactivation and, in turn, the activation of E2F TFs. Importantly, D-type cyclins are also believed to link cell cycle activity with developmental and environmental stimuli and consistently, the expression of several CYCD genes was shown to be regulated by nutritional and hormonal signals (reviewed by de Jager *et al.,* 2005). The expression of Arabidopsis *CYCD3;1* is induced by both sucrose and cytokinins and, in agreement with this, *CYCD3;1* over-expression in cell cultures overcomes G1 phase arrest in sucrose starved cells (Riou-Khamlichi *et al.,* 1999, 2000; Oakenfull *et al.,* 2002; Menges *et al.,* 2006). In a separate study, CYCD3;1, CYCD3;2 and CYCD3;3 were shown to be essential for cell cycle activation in the root apical meristem during germination (Masubelele *et al.,* 2005). Another important class of G1 regulators is the Kip related proteins (KRPs) which, through their interaction with CDKA and CYCD, repress cell division. Similar to CYCD proteins, KRPs were shown to be transcriptional regulated and to play a role in modulating cell cycle activity upon development and environmental stimuli (Wang *et al.,* 1998, 2000; DeVeylder *et al.,* 2001; Jasinski *et al.,* 2002; Zhou *et al.,* 2002).

Despite the obvious participation of transcription in the control of the cell cycle, for most of the TFs that affect cell proliferation either spatially, temporally or quantitatively during development or environmental responses, it is not clear whether they directly regulate core cell cycle genes such as *CYCDs* and *KRPs* (Gegas and Doonan, 2006). Exceptions include a membrane bound NAC TF named NTM1 that was shown to act upstream of *CYCD3;1*, *KRP2* and *KRP7* (Kim *et al.,* 2006); an AP2 TF named ENHANCED SHOOT REGENERATION 2 (ESR2) whose direct targets include *CYCD1;1* and *KRP4 (*Ikeda *et al.,* 2006*)*; and the AP2 TF AINTEGUMENTA (ANT) that maintains the meristematic competence of cells by sustaining expression of *CYCD3;1* (Mizukami and Fischer, 2000). Importantly we gained evidence for involvement of DOF TF OBP1 in the regulation of cell cycle activity at the G1 control point by targeting the expression of core cell cycle genes (*CYCD3;3* and *KRP7*), other TFs and components of the replication machinery in a developmental specific manner.

In Arabidopsis, family of DNA-binding-with-one-finger (DOF) TFs contains 37 members playing divergent roles in plant specific processes. Their highly conserved N-terminal DOF region acts as a DNA-binding domain and corresponds to a conserved DNA *cis*-element

(A/T)AAAG or its complementary inverse sequence (summarized in Yanagisawa, 2002, and Yanagisawa, 2004). The DOF TF OBF4 BINDING PROTEIN 1 (OBP1) was, as its name suggests, previously identified in a screen for protein partners of bZIP transcription factor OBF4 (Zhang *et al.,* 1995). *In vitro* OBP1 stimulates OBF4 binding to *ocs* elements found in the cauliflower mosaic virus 35S (CaMV) and plant glutathione S-transferase (*GST6*) promoters (Chen *et al.,* 1996). Subsequently two other un-related DOF transcription factors, named OBP2 and OBP3, were shown to have similar qualities. They all bind OBF4 in GST pull-down assays and enhanced OBF4 binding to the *ocs* element (Kang and Singh, 2000). These initial *in vitro* based findings have been followed by more detailed characterisation of *OBP2* and *OBP3*. Both genes are phloem specific and when constitutively over-expressed result in a strong phenotype characterised by severely reduced growth (Ward *et al.,* 2005; Skirycz *et al.,* 2006). OBP3 was characterised as a novel component of light signalling; a positive regulator of phyB mediated inhibition of hypocotyl elongation and a negative regulator of cry1 mediated cotyledon expansion (Ward *et al.,* 2005). OBP2 in turn is involved in regulation of indole glucosinolate metabolism (Skirycz *et al.,* 2006). Significantly none of these studies provided *in vivo* evidence of *GST6* regulation.

5.3 Results

5.3.1 Identification of OBP1-regulated biological processes by profiling technologies.

Co-expression analysis is a proven tool to elucidate possible biological function (Gachon *et al.,* 2005; Persson *et al.,* 2005). Therefore, to identify biological processes that may be regulated by OBP1 we utilised the Arabidopsis co-expression tool (ACT) (Manfield *et al.,* 2006). Out of the top 48 genes whose expression positively correlates with *OBP1*, the majority can be assigned to DNA/RNA metabolism, cell division and organization (Table S1). These include genes encoding for DNA polymerase α, MCM factors, DNA mismatch repair protein, A-type cyclin and kinesins.

To further characterize OBP1, we followed a functional genomics approach using transcript profiling with Affymetrix near-full genome ATH1 arrays and metabolite profiling by GC-MS. For this approach we used a dexamethasone (DEX)-inducible system in which *OBP1* fused to a glucocorticoid receptor (GR) domain was placed under the control of the CaMV 35S promoter (Lloyd *et al.,* 1994). DEX-treatment induces nuclear targeting of the OBP1-GR fusion protein which thus causes activation of target genes. As expression is constitutive the transcript level of the chimeric gene itself remains unchanged.

To identify candidate genes, a single *inducible over-expression (IOE)-OBP1* line was compared to an empty-vector control line 10h after DEX induction using expression profiling. In total 631 transcripts were up-regulated and 842 down-regulated (2-fold cutoff) (Table S2-S3). Over representation analysis using PageMan (Usadel *et al.,* 2006) revealed significant enrichment of cell cycle and cell organization genes among up-regulated transcripts and cell wall modification and signaling receptor kinases among down-regulated transcripts (data not shown). We also compared our results to publicly available datasets. In total, 190 of the up-regulated transcripts, ~6-fold more than expected by chance, were previously shown to oscillate during the cell cycle with the majority peaking during the G2 and M phases (Menges *et al.,* 2003). Importantly, the same study showed that *OBP1* expression peaked during the G1 phase (Figure 1a-b, Table S4). Of 131 genes assigned as high confidence proliferation genes based on the their expression in the root apical meristem, young proliferating leaves and cell cultures (Beemster *et al.,* 2005), 85 were up-regulated in our study (Table S5). Furthermore, out of the 87 core cell cycle genes (Menges *et al.,* 2005; Wang *et al.,* 2004; Vandepoele *et al.,* 2004) represented on the Affymetrix ATH1 array, 24 were induced (Figure 1c, Table S6). Among down-regulated transcripts over-representation analysis revealed significant enrichment of genes encoding for cell wall loosening enzymes (Table S6). These data urged us to check other growth markers (ribosomal proteins, components of TOR signaling, TCP TFs, other TFs such as

ARGOS and AINTEGUMENTA) (Ingram and Waites, 2006), but except for two TCP TFs we detected no differences.

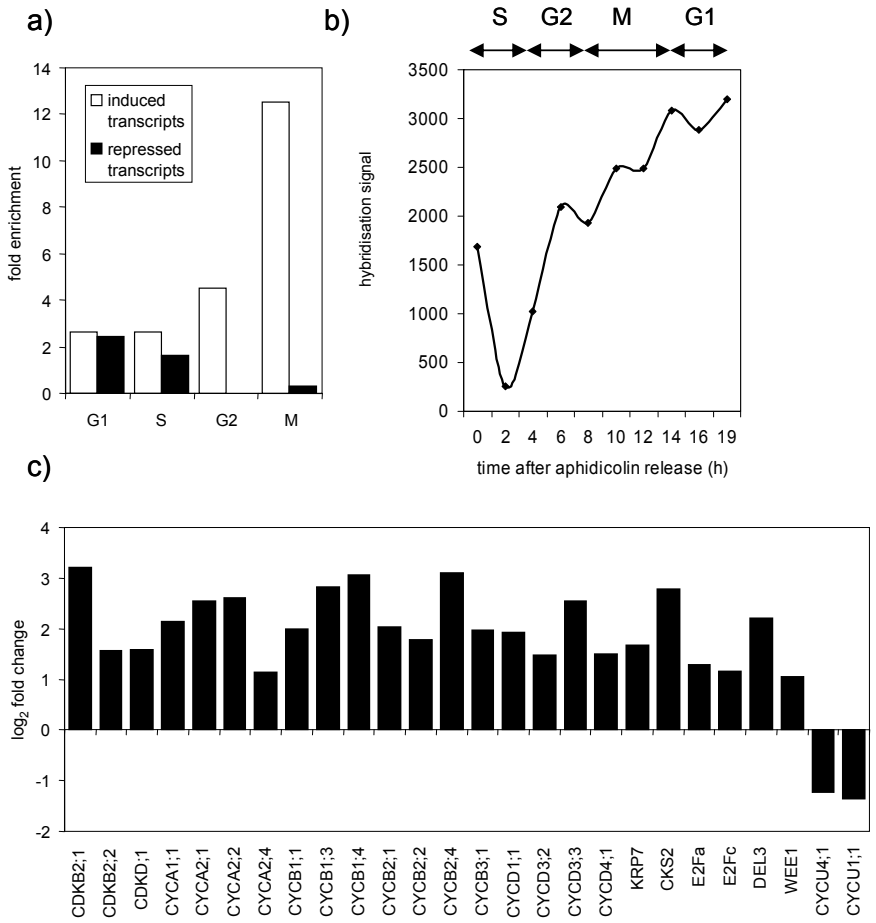


Figure 1. *OBP1* expression is G1phase specific and inducible *OBP1* over-expression regulates many cell cycle related genes. a) Fold enrichment of genes that expression was shown to oscillate during the cell cycle (Menges *et al.,* 2003) among transcripts up- and down-regulated by OBP1 activation. b) *OBP1* expression peaks in G1 phase in cell cycle progression, aphidicolin block/release experiment (Menges *et al.,* 2003). c) Up- or down-regulated core cell cycle genes as revealed by transcript profiling of *IOE-OBP1* lines in comparison to empty vector control plants 10h after DEX induction. Data are from one biological experiment; each sample was pooled from three fully developed Arabidopsis rosettes.

Subsequently we confirmed our microarray data using Q-RTPCR for 17 up-regulated genes in two independent *IOE-OBP1* lines in three biologically independent experiments. Comparison was made between mock- and DEX-treated plants and the effect of DEX was evaluated by including an empty vector control line that was sprayed with DEX (Table 1). Expression of 15 out

of the 17 selected genes was up-regulated in both lines and for 14 genes this induction was significant. As GC-MS metabolite profiling is a useful tool to identify affected biological processes we sought to use it to complement our transcript analysis. However, we were unable to detect any prominent changes in samples harvested 10 and 24h after DEX induction. Together, our data imply OBP1 involvement in cell proliferation.

Table 1. Expression changes of selected genes in DEX-inducible *OBP1* lines. Transcript levels were measured using Q-RTPCR in *IOE-OBP1* (lines IOE#4, IOE#7) and empty-vector control plants (Control) 10h after DEX induction. Rosette samples were taken from plants prior to bolting and pooled from three plants, the experiment was repeated three times under long day conditions (data are means ± STDEV, *n* = 3). Bold fond and grey shading indicate values that are significantly different from the respective controls (dark grey, $p < 0.05$; ligh grey, $p < 0.1$; Student's t-test). ΔCt = Ct (gene) – mean Ct (house-keeping genes); ΔΔCt= ΔCt (mock) – ΔCt (DEX). The last column indicates the expression changes determined by Affymetrix expression profiling 10h after DEX induction.

		Q-RTPCR; (10h) ΔΔCt (log_2)		Affy (10h) (log_2)
Gene Name	**Control**	**IOE#4**	**IOE#7**	**IOE#7**
MCM2	-0.09±0.24	**1.41±0.36**	**2.16±0.95**	1.88
MCM5	-0.25±0.42	0.62±0.13	0.15±0.41	1.39
SNC1	-0.58±0.32	**1.57±0.36**	-0.39±2.53	2.44
Unknown At5g17160	-0.91±0.54	**2.71±0.42**	**2.90±0.11**	3.82
Kinesin At5g33300	-0.37±0.24	1.21±0.77	**2.76±0.11**	2.82
Kinesin At3g44050	-1.47±1.16	**1.46±0.60**	**2.24±0.24**	4.01
CKS2	-0.56±0.40	**1.71±0.69**	**2.88±0.75**	2.78
CDKB2;1	-0.72±0.38	**2.02±0.35**	**4.48±1.65**	3.21
CYCA2;2	-0.70±0.61	1.84±1.35	**2.94±0.56**	2.62
CYCB1;3	-0.49±0.29	**1.68±0.37**	**2.93±0.23**	2.83
CYCB2;4	-0.95±0.90	1.09±0.49	**3.08±0.30**	3.11
CYCD3;2	-0.46±0.45	**0.82±0.05**	2.00±1.06	1.48
CYCD3;3	-0.70±0.29	**2.60±0.30**	**2.22±0.76**	2.56
CYCD4;1	-0.38±0.40	**0.67±0.22**	1.05±0.50	1.51
DEL3	-1.12±0.82	**1.71±0.22**	**2.39±0.68**	2.20
E2Fa	-1.03±0.69	**1.00±0.47**	0.81±0.10	1.29
AtDOF2;3	-0.54±0.58	**2.55±0.32**	**3.11±0.61**	1.56

5.3.2 Identification of OBP1 target genes

The large number of expression changes 10 hours after DEX induction suggested that these might be secondary targets of OBP1. Therefore, to identify more direct OBP1 targets we profiled selected genes 5h after induction. Given the probable involvement of OBP1 in cell cycle regulation, we designed Q-RTPCR primers for 82 core cell cycle genes, 90 cell cycle specific TFs, *GST6* and two replication licensing factors *MCM2* and *MCM5*. Among these we identified eight genes consistently up-regulated 5 and 10h after induction; four TFs (*MYB88*, *TAZ-*

At5g67480, *MYB-like* At2g42970 and *AtDOF2;3*), two core cell cycle proteins (D-type cyclin; *CYCD3;3*, and Kip-related protein; *KRP7*) and two replication licensing factors (*MCM2* and *MCM5*) (Table 2a). To further refine our target list we also measured the expression of these genes in *35S-OBP1* and *RNAi-OBP1* lines (Table 2b). In general, fewer genes were regulated in these lines than we identified using *IOE-OBP1*. As the *35S-OBP1* lines showed a strong phenotype (see section 5.3.3) we rather decided to focus our molecular studies on *CYCD3;3* and *AtDOF2;3* as we detected lower expression in *RNAi-OBP1* plants (Table 2b).

Table 2. Expression changes of selected genes in plants with modified OBP1 expression. a) Transcript levels were measured using Q-RTPCR in *IOE-OBP1* (lines IOE#4, IOE#7) and empty vector control plants (Control) 5h after DEX induction. (data are means ± STDEV, *n* = 3, or means, *n* = 2). Biological replication was from individual plants within a single experiment for the short day grown plants and between three experiments for the long day grown plants. For reference, the last column shows expression changes as determined by Affymetrix expression profiling 10h after DEX induction. b) Transcript levels were measured using Q-RTPCR in *RNAi-OBP1* (lines #1, #2 and #3) and *35S-OBP1* (lines #1, #6 and #9) plants. (data are means ± STDEV, *n* = 3, or means, *n* = 1 or 2). For *RNAi-OBP1* biological replication was between independent experiments. For experiment 2 (Ex2) biological replication was within the experiment from individual *35S-OBP1* plants. a-b) All samples were whole rosettes prior to bolting and unless otherwise stated samples were pooled from 3 plants. Bold font and grey shading indicate values that are significantly different from the respective controls (dark grey, $p < 0.05$; light grey, $p < 0.1$; Student's t-test). ΔCt = Ct (gene) – mean Ct (house-keeping genes)); ΔΔCt= ΔCt (mock) – ΔCt (DEX) or ΔΔCt= ΔCt (control) – ΔCt (transgenic).

a)	Q-RTPCR; SHORT DAY (5h)			Q-RTPCR; LONG DAY (5h)			Affy (10h)
	ΔΔCt (log_2)			ΔΔCt (log_2)			(log_2)
	Control	IOE#4	IOE#7	Control	IOE#4	IOE#7	IOE#7
CYCD3;3	0.54±0.80	**1.58±0.96**	**1.45±0.71**	-0.24±1.14	**1.87±0.64**	2.33	2.56
KRP7	-0.07±0.90	**1.02±0.66**	**1.07±0.17**	-0.34±1.41	**2.33±0.77**	1.84	1.67
MYB88	-0.63±0.99	**1.53±0.62**	**0.97±0.47**	-0.12±0.47	**2.75±0.77**	1.71	1.94
DOF2;3	-0.05±0.65	**1.21±0.84**	**1.01±0.45**	0.13±0.54	**2.03±0.65**	0.80	1.56
MYB-like	-0.54±0.72	0.53±0.53	**0.55±0.19**	-0.24±0.44	**0.59±0.32**	0.81	0.7
TAZ (5g67480)	-0.11±0.35	**0.79±0.68**	**0.64±0.32**	-0.35±0.50	2.16±2.06	1.56	1.36
MCM5	-0.22±0.96	**0.82±0.28**	**0.89±0.22**	-0.22±0.38	**0.95±0.18**	1.95	1.39
MCM2	-0.11±1.46	**1.27±0.55**	**1.14±0.57**	-0.22±0.45	**1.77±0.48**	1.17	1.88

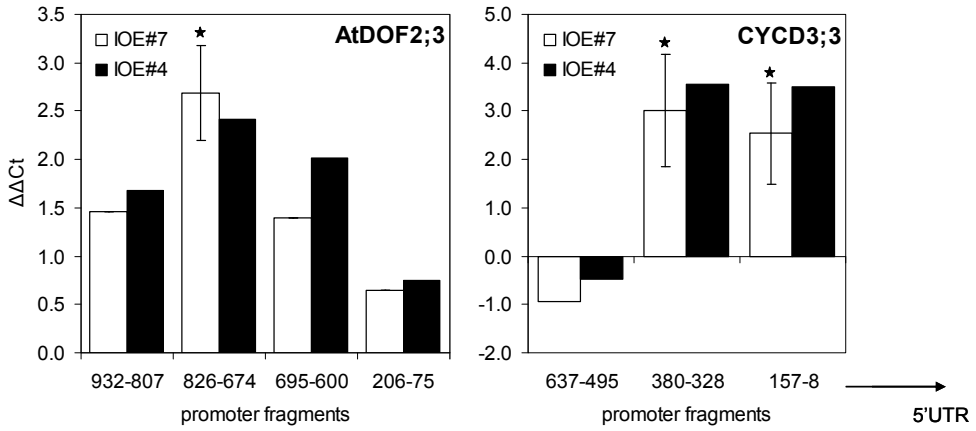
b)	RNAi-OBP1			35S-OBP1				
	ΔΔCt (log_2)			ΔΔCt (log_2)				
	RNAi#1 (n=1)	RNAi#2 (n=2)	RNAi#3 (n=3)	35#1 Ex1	35#6 Ex1	35#9 Ex1	35S#1 Ex2	35S#6 Ex2
CYCD3;3	-1.72	-0.40	**-1.09±0.70**	0.43	-0.23	1.26	-0.32±0.98	0.07±1.42
KRP7	-0.02	-0.01	-0.03±0.10	0.73	1.42	1.16	**0.84±0.47**	**0.88±0.26**
MYB88	-0.23	-0.01	**-0.27±0.20**	-0.24	1.87	2.97	**1.15±0.33**	**1.21±0.33**
DOF2;3	-2.62	-0.89	**-1.57±1.39**	1.65	2.82	1.90	**1.45±0.54**	**1.97±0.60**
MYB - like	-0.86	-0.24	-0.41±0.34	0.87	2.29	1.79	0.22±0.11	0.06±0.14
TAZ (5g67480)	-1.31	-0.20	-0.48±0.67	0.73	0.75	1.26	-0.45±0.50	-1.07±1.29

MCM5	-0.19	-0.17	0.14± 0.42	-1.03	0.76	0.96	0.51±0.46	0.41±0.15
MCM2	-0.54	0.28	0.15± 0.38	0.29	1.40	1.56	0.59±0.60	0.86±0.10
OBP1	-2.95	-0.93	**-1.11± 0.86**	3.97	8.28	8.22	**6.99±0.22**	**7.14±0.23**

To establish whether the regulation of *CYCD3;3* and *AtDOF2;3* measured in transgenic lines with altered *OBP1* expression was due to direct OBP1 binding to their promoter sequences we performed chromatin immuno-precipitation (ChIP) experiments using an anti-GR antibody. We designed primers at intervals along the promoters of these genes and measured their abundance using Q-RTPCR. Specifically, we compared the abundance of three *CYCD3;3* and four *AtDOF2;3* promoter fragments in samples from control plants and two independent *IOE-OBP1* lines 5h after DEX application. We detected enrichment for both genes (Figure 2a) with highest enrichment for fragments spanning ~150 and ~400bp for *AtDOF2;3* and *CYCD3;3* promoters. Given the enrichment patterns for the different fragments these regions most likely contain the *cis*-element(s) bound by OBP1. DOF TFs have been shown to bind to the sequence CTTT(T/A) or its complement and have a higher affinity to tandem sites (Yanagisawa and Schmidt, 1999; Plesch *et al.,* 2001). The *AtDOF2;3* fragment contains three DOF binding sites (Figure 2b), whilst the longer *CYCD3;3* fragment contains 13 (Figure 2c) and both contain tandem DOF sites. In addition, using EMSA we confirmed OBP1 binding to the consensus DOF element (we used enriched *AtDOF2;3* promoter fragment containing tandem DOF sites) and again demonstrated that OBP1 had higher affinity for DNA fragments with tandem DOF sites (Figure 2d).

Additional experiments are required to test which of the DOF sites are functional. In the absence of such experiments we sought alternative data to support the putative function of the identified binding sites. Phylogenetic sequence conservation has been demonstrated for functional TF binding sites (Koch *et al.,* 2001; Hong *et al.,* 2003). Therefore, we looked at the promoter region that was enriched in ChIP experiments in the poplar ortholog of *AtDOF2;3* and found a tandem DOF element in approximately the same position relative to the translational start codon as for the Arabidopsis gene (Figure S1).

a)



b)

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CACAAATCCATCCAAATGTTCTTTGAAAAGAAAATATGTATGGGCCGGGGCAAGACTCCTAGGTATCTAGAGAGTTGCATTTCAAACCTAATGATAATAGATTATTT
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CCCTGAAATTCTAATTCGTTAATAATCCATGAACATAGACTCAAATTCGGATGTGAATAGATGGATCATGTTTATAGAATCTCTTTCAAGTAAATTTAATCTTATTT
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TGCACGTTTTCATG
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c)

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GTTACATAAAGAAAAATTAAGTAACGTAGTTTACTAATAAAGCGCAAGCGTTTTAACAGAACGAGGTCCTTTTTTCCTAGTTAATTATGTTTTTGTTTTTTCTAATG
TTTATGTCAGCTCGTTGACCATATAAAGCTTCCCATTCGTCCACGTGGATTTTCCTCTGACATATATGACCCCACGGGTGATTTTATATGATTATATATATCAATTT
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TCATG
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d)

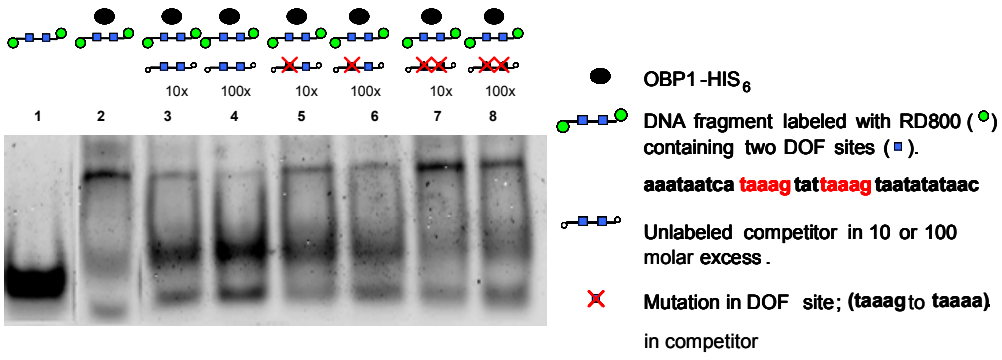


Figure 2. OBP1 binds to promoter fragment of *CYCD3;3* and *AtDOF2;3* genes. a) DEX induced *IOE-OBP1* and control plants were used for ChIP experiments. Abundance of *CYCD3;3* and *AtDOF2;3* promoter fragments was determined using Q-RTPCR. The graph shows $\log_2$ enrichment of the promoter fragments in two *IOE-OBP1* lines compared to control plants in three independent experiments. Data were normalised using genomic primers for *UBQ10*. (data are means ± STDEV, *n* = 3, or means, *n* = 2). Asterisks indicate values that are significantly different from the respective controls ($p < 0.05$, Student's t-test). (b-c) Approximately 1kb promoter fragments of (b) *AtDOF2;3* and (c) *CYCD3;3* genes. Sequence features are highlighted: ATG, green; 5' UTR, bold; putative DOF elements (AAAG, CTTT, [A/T]AAAG,

CTTT[A/T]), grey, and enriched fragment, blue. d) Fluorescent electrophoretic-mobility shift assay was performed using OBP1-His_6 protein, *AtDOF2;3* promoter fragment labelled with fluorescent probe RD800 and unlabelled competitor sequences with or without mutated DOF binding sites. 1 – RD800 labelled DNA fragment. 2 – OBP1 protein and labelled DNA fragment. Note distinct shift indicating binding. 3, 4 – OBP1 protein, labelled DNA fragment and excess competitor. Note proportional shift disappearance. 5, 6 - OBP1 protein, labelled DNA fragment and excess of competitor containing one mutated DOF element. Note lower proportional shift disappearance compared to 3 and 4, indicating higher OBP1 affinity to tandem DOF sites. 7, 8 - OBP1 protein, labelled DNA fragment and excess of competitor containing two mutated DOF elements. Note that result similar to 2 indicating the requirement of DOF elements for competition.

5.3.3 OBP1 – cell proliferation and cell expansion

As molecular data indicated the likely involvement of OBP1 in cell cycle regulation we decided to utilise cell cultures as a model to gain more functional insight. Cell suspension cultures have the advantage that cell cycle processes can be studied in a homogeneous culture independently of cellular differentiation and developmental events. We selected Arabidopsis MM2d cells as they can be easily synchronised (Menges and Murray, 2002) and transformed them with the *IOE-OBP1* construct. For further analysis we picked two independent lines with high *OBP1* expression as determined using Q-RTPCR. Initially, we analysed cell number, wet weight and cell size during standard culture growth, for seven days after subculture. All the comparisons were made between DEX induced and mock treated lines and the DEX effect was evaluated by using wild-type cultures. The most striking observation was a significant reduction in both wet weight and cell size within 24h after DEX application (Figure 3a-3d and 3h-3k). In all experiments the number of cells increased significantly more in DEX versus mock grown *IOE-OBP1* lines but only in the first 24h following induction (Figure 3e-3f). Later the rate of cell division was similar or slower. These data indicated that the first 24h after DEX induction were most informative.

To investigate if the increased rate of cell division was dependent on the culture growth phase we induced cultures with DEX straight after subculture and during the exponential growth phase. Increased cell division in comparison to mock treated controls was observed at both phases (Figure 3g). To more precisely determine the timing of the observed differences we took measurements every 4 h for 20 h after DEX induction. These data revealed that cell number increased approximately 12 h after induction and subsequently the rate of division slowed down (Figure 4a-4c). This indicates that OBP1 induction must shorten the duration of the cell cycle and so we decided to use flow cytometry to investigate cell cycle phase distribution. Within 4h of DEX induction we measured a higher percentage of S phase nuclei in induced samples at the

expense of G1 nuclei. This suggests that the increase in cell number may be due to a shortening of the G1 phase (Figure 4d-4e). To get more direct evidence we performed sucrose starvation/re-addition experiments. Sucrose removal leads to arrest primarily in G1 phase whilst re-addition allows cells to progress through the cell cycle. In order to study OBP1 function, sucrose re-addition was accompanied by DEX induction and we compared the phase distribution of nuclei in samples harvested every 2-3h for 15h. In DEX induced cultures, there was a steeper decline in the proportion of G1 nuclei and the S phase peak occurred earlier, after 6h instead of 8h. Consistent with the previous experiment, this indicates OBP1 activation caused a faster transition from G1 to S phase. In addition, induction also caused the proportion of G2 phase nuclei to decline earlier and was accompanied by a sharp increase in G1 phase nuclei after 12-15h. This indicates that the effect of OBP1 on the G1 duration is perpetuated through the G2 phase, resulting in earlier division (Figure 5).

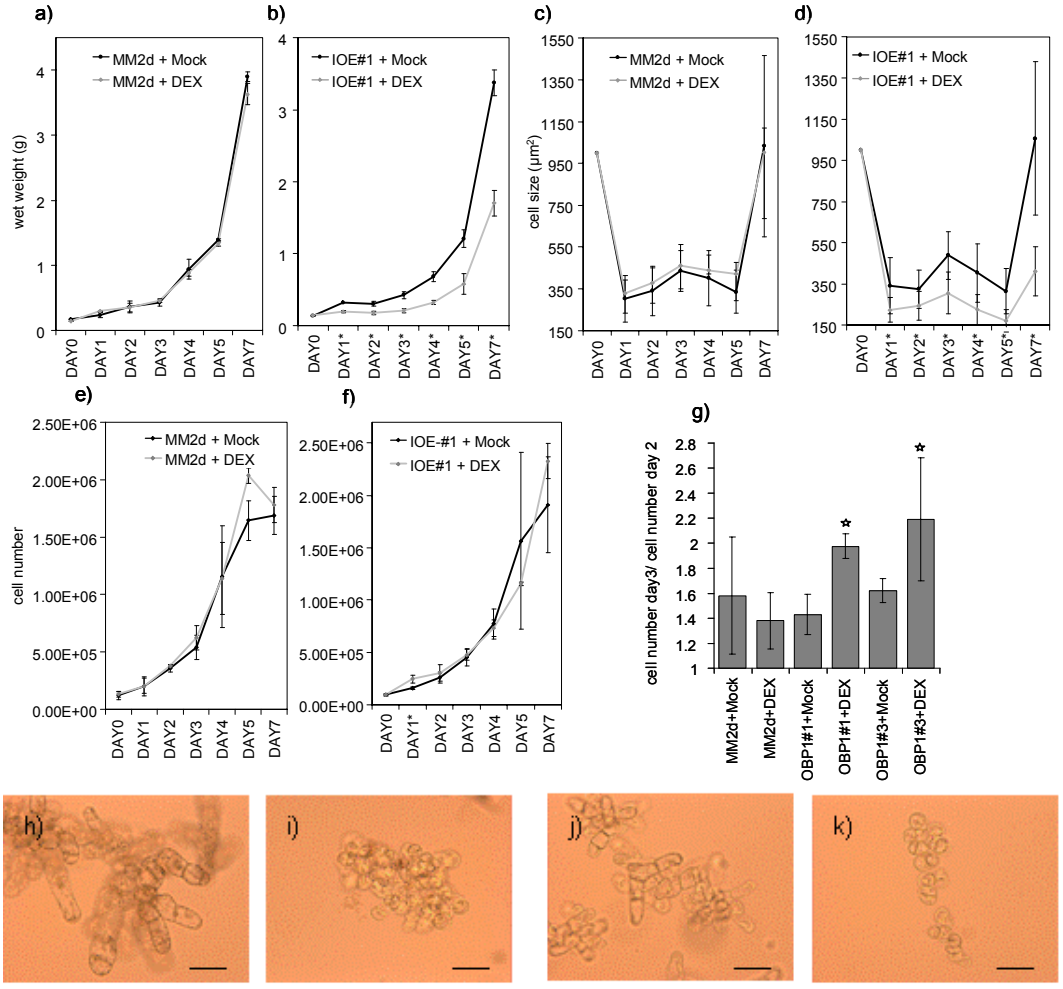


Figure 3. Cell growth and proliferation are affected by OBP1 over-expression. Early stationary phase cells (7 days after subculture) of MM2d wild-type and *IOE-OBP1* transgenic lines (IOE#1 and IOE#3) were

transferred into fresh medium and sampled as indicated. For induction experiments a freshly prepared 200 ml culture was split into two 100ml cultures, with one DEX-induced (5 µM) and the other mock-induced (0.005 % EtOH). (a, b) Determination of biomass, (c, d) cell size, and (e, f) cell number (per 1ml of culture) during seven day time course. (data are means ± STDEV, *n* = 3 independent experiments). (g) The ratio of cell number calculated between day 2 and day 3 of exponential growth. MM2d wild-type and *IOE-OBP1* transgenic cell cultures lines (IOE#1 and IOE#3) were induced two days after subculture (data are means ± STDEV, *n* = 3 flasks within one experiment). Effect of *OBP1* over-expression on cell size. (h, i) *IOE-OBP1#1* cells that were grown for seven days (h) without or (i) with DEX. (j, k) *IOE-OBP1#3* cells that were grown for seven days without (j) or with DEX (k).

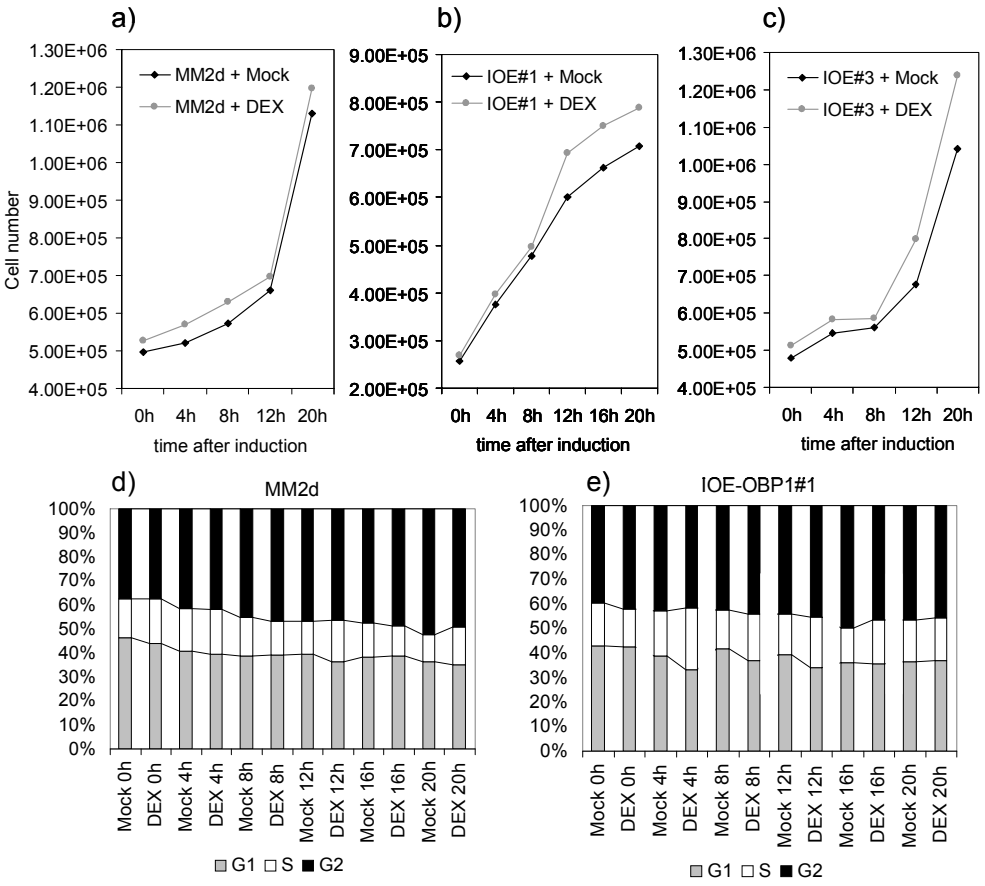


Figure 4. OBP1 affects the rate of cell proliferation. Exponentially growing (2 days after subculture) MM2d wild type and *IOE-OBP1* transgenic cell cultures (lines IOE#1 and IOE#3) were either DEX induced (5 µM) or mock induced (0.005 % EtOH). Data are mean from two independent flasks in one experiment. a, b, c) Cell number was counted every 4h for 20h. Cell number is given per 1 ml of culture. d, e) Phase distribution of nuclei measured by flow cytometry.

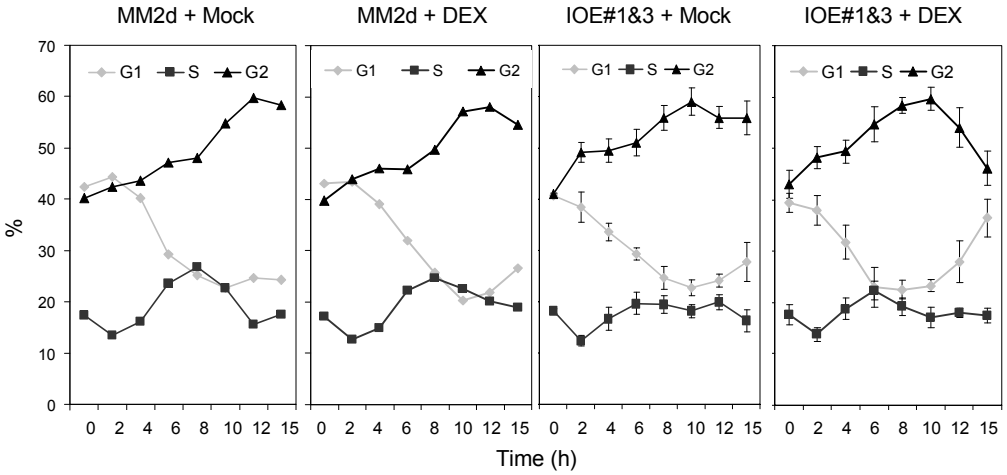
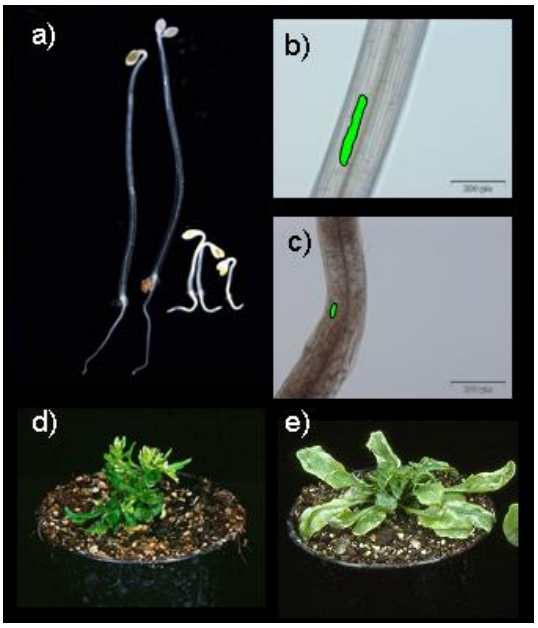


Figure 5. OBP1 shortens the length of the cell cycle. A sucrose starvation/re-addition experiment was performed as described by Menges *et al.* (2002). Sucrose re-addition was accompanied by mock (0.005 % EtOH) or DEX (5 µM) induction. Data for *IOE-OBP1* transgenic cell cultures (lines IOE#1 and IOE#3) are means ± STDEV from four independent flasks in one experiment. Phase distribution of nuclei measured by flow cytometry.

To determine how the effects of OBP1 on cell proliferation and expansion influence growth and development, we performed phenotypic analysis of OBP1 transgenic plants. Constitutive over-expression resulted in drastically smaller plants compared to wild-type. Seedlings were characterised by shorter hypocotyls (especially pronounced when grown in the dark) and stunted roots (Figure 6a-6c). Later in development *35S-OBP1* plants developed smaller, curly leaves that were occasionally lobed (Figure 6d) and characterised by un-branched trichomes (data not shown). Main roots were shorter with less lateral roots being formed (Figure 6f). Flowering was delayed and the main inflorescence was much shorter (Figure 6d). Also shorter were stamens resulting in decreased fertility and poor seed setting. We could recreate the identical seedling phenotype for *IOE-OBP1* plants grown on DEX. Also rosettes sprayed with DEX develop a similar phenotypes within 3-4 days of induction; developing leaves became curly and eventually smaller whilst older leaves often became yellow and occasionally died (Figure 6e). In contrast, we observed no obvious visible phenotype for *RNAi-OBP1* plants, which may be due to the incomplete silencing (Table 2b).

As there are often interactions between changes in cell size and number, we measured cell size and number in cotyledons and fully developed rosette leaves. Similar to the situation in cell cultures epidermis cell size was approximately 50-70% that of the control (Figure 6f). We calculated cell number from cell size and total cotyledon/leaf area and could detect a significant

increase in number of cells in cotyledons but a decrease in true leaves (Figure 6f). As alterations in cell size are sometimes associated with changes in ploidy levels and link between D-type cyclins and endoreduplication was recently proposed (Dewitte *et al.,* 2007) we additionally analysed nuclei ploidy distribution. We selected leaves and dark and light grown hypocotyls and measured a significant reduction of ploidy (Table S6).



f)

Epidermal cells			
	Control	**35S#1**	**35S#6**
Adaxial epidermis - Cotyledons			
Total cells	310.13±14.7	**450.27±66.6**	**380.93± 45.9**
Cell area (μm²)	10393.7± 3054	**8061.1± 2602**	**8159.7± 2765**
Adaxial epidermis - Leaves			
Total cells	4007.5±501.7	**3286.2±297.4**	**3397.8±195.4**
Cell area (μm²)	7168.1±2871	**3083.3±1427**	**5641.2±2286**
Roots			
Total Lateral Roots	6.51±4.19	**2.28±2.01**	**1.12±1.29**
Root Length (mm)	22.63±8.48	**16.57±7.97**	**11.36±6.35**

Figure 6. Phenotypic analysis of *OBP1* over-expression plants. (a) Seedlings of control (left) and *IOE-OBP1* (right) plants grown on 5μM DEX and in dark. b) Hypocotyl of the control, dark grown seedling (bar=200 μm). c) Hypocotyl of *IOE-OBP1* dark grown seedling; note the small cells (bar=200 μm). d) Flowering *35S-OBP1* plant. e) *IOE-OBP1* plant a week after DEX application. f) Analysis of epidermal cells of cotyledons and the sixth rosette leaf of empty vector control and *35S-OBP1* plants (data are mean ± STDEV, *n* = 4-6). Root length and lateral root number were calculated for ten day old seedlings using stereomicroscope MZ 12,5 (Leica) and cell^P Software (Olympus) (data are means ± STDEV, *n* = 30-50). Bold font and shading indicate values that are significantly different from the respective control plants ($p < 0.05$; Student´s t-test).

5.3.4 Biological context of OBP1 action

To learn more about the biological role of OBP1 action we examined its expression pattern. Mining publicly available microarray data was supported by the analysis of lines expressing the β-glucuronidase (GUS) reporter gene under the control of ~2-kb of the *OBP1* promoter. During embryogenesis strongest *OBP1* expression was found in the apical part at the globular stage, in

the emerging cotyledons at the heart stage and in the cotyledons at the torpedo stage (Figure 7a-7c). GUS staining was absent from the dormant embryos, appeared again few hours after the start of seed imbibition and stayed strong in the root (RAM) and shoot (SAM) apical meristem and the cotyledon tips of germinating seedlings (Figure 7d-7f). Expression in the RAM disappeared later in development but was visible in lateral roots initials (Figure 7g). In contrast, *OBP1* expression in the SAM was high throughout development with strongest GUS staining observed in stipules (Figure 7h). Moderate GUS staining was also observed for the whole leaf in very young leaves but later first became restricted to the leaf tip and occasionally hydathodes (Figure 7i) and then disappeared completely at the senescence stage. Our GUS data are consistent with published expression profiling data on leaf development (Beemster *et al.,* 2005). In the stem GUS staining appeared only in the vasculature of the cut ends (Figure 7j-k). High *OBP1* expression was also characteristic for axiliary nodes and nectaries of flower (Figure 7j and 7l). Microarray data indicated *OBP1* expression was highest in callus and in cell cultures (during exponential phase of growth) and we could confirm it by GUS analysis (Figure 7m). In contrast to the diverse developmental expression pattern there were only few treatments that affected *OBP1* expression. Most pronounced *OBP1* induction was identified in cell cultures after addition of the cell wall disturbing agent isoxaben (Manfield *et al.,* 2004).

Subsequently using the Genevestigator meta-analysis tool (Zimmermann *et al.,* 2004) we compared the tissue expression pattern of *OBP1* and its early target genes (*CYCD3;3, KRP7, MYB-like*, *AtDOF2;3,MYB88,TAZ, MCM2* and *MCM5*). The expression patterns of these genes overlapped well, with relatively high expression in cell cultures, callus, shoot apex, radicle and root (Figure S2). Considering the expression of *OBP1* in the root apex of germinating seedlings and a recent publication implicating cell cycle activation in radicle protrusion (Masubelele *et al.,* 2006), we investigated the expression of these genes in seed germination expression profiling data. Expression of several D-type cyclins including *CYCD3;3* peaked shortly after the end of seed stratification, followed by other components of the core cell cycle machinery and was proceeded by increased *OBP1* expression (Masubelele *et al.,* 2006). This was confirmed by two other experimental data sets (AtGenExpress project) in which expression of *OBP1* increased 3h after imbibition, followed by its early targets (*CYCD3;3, MYB-like TF*, *AtDOF2;3, MCM2* and *MCM5*) (Figure S3). Importantly transcripts of *CYCD3;3, MYB-like TF* and *AtDOF2;3* are relatively highly abundant in the radicles of young seedlings.

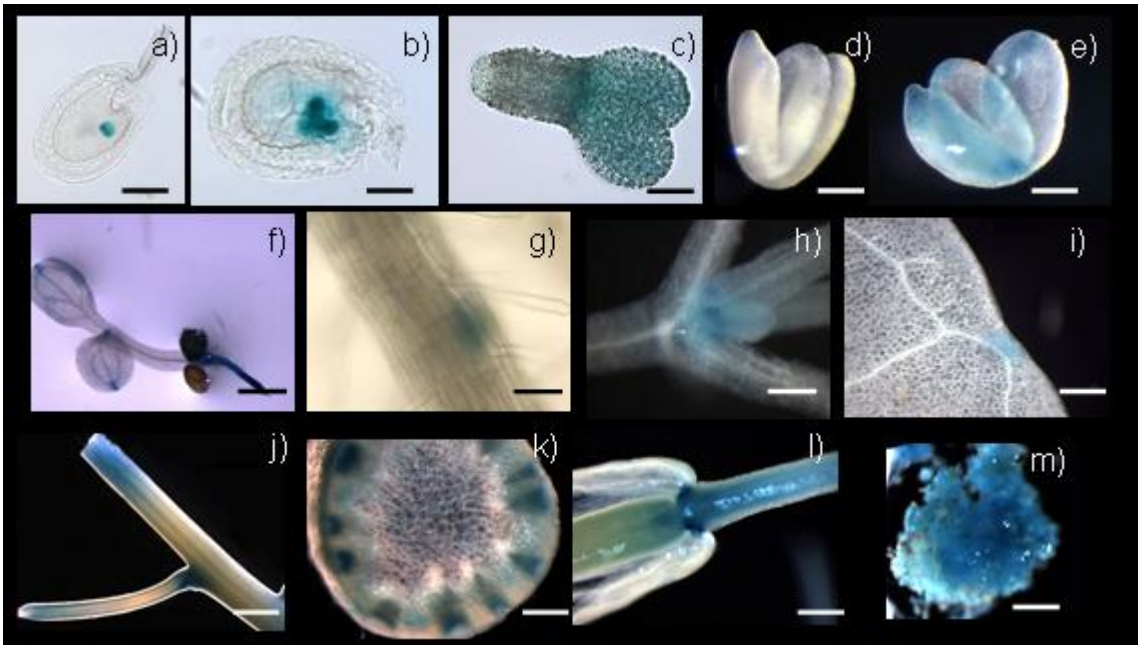


Figure 7. *OBP1* expression pattern defined by the analysis of *OBP1-GUS* lines. a) Embryo at the globular stage (bar=75µm). b) Embryo at the heart stage (bar=75µm). c) Embryo at the torpedo stage (bar=75µm). d) Mature embryo of a dormant seed (bar=75µm). e) Embryo at the end of stratification (bar=75µm). f) Seedling 2 days after germination (bar=1mm). g) Lateral root initial (bar=200µm). h) Shoot apical meristem of a one week old seedling (bar=1mm). i) Hydathode (bar=1mm). j) Cut stem segment with axillary node (bar=2mm). k) Hand cut stem section (bar=200 µm). l) Pedicle; nc – nectaries (bar=1mm). m) Callus obtained from MM2d cells transformed with *OBP1-GUS* construct.

5.3.5 Factors up-stream of OBP1

To identify putative regulatory factors up-stream of OBP1 we performed a search of literature and available expression data. Interestingly, *OBP1* was among 51 targets of the AP2/ERF TF Enhanced Shoot Regeneration 2 (ESR2) identified using expression profiling of estradiol inducible ESR2 lines (Ikeda *et al.,* 2006). It was therefore of interest to investigate if there was further evidence to support this interaction.

As mentioned previously, functional regulatory elements are often conserved between species (Wasserman and Sandelin, 2004). We looked at the *OBP1* promoter in an attempt to identify potential ESR2 binding sites. Although the usual consensus binding site of AP2/ERF TFs is [A/T]GCCGCC, a screen for possible binding sites of the close ESR2 homolog ESR1 retrieved also the sequence [A/T]GCCGAC (Banno *et al.,* 2006). Significantly, not only is the latter of these sequences present in the promoter of Arabidopsis *OBP1*, it is also conserved in the promoters of *OBP1* orthologs from *Brassica oleracea* and poplar (Figure S4).

Given that molecular and sequence data support the regulation of *OBP1* by ESR2, we investigated if physiological data was also in agreement. Constitutive activation of *ESR2* results in a dwarfish phenotype related to smaller and fewer cells and manifested by small epinastic (curved downwards) leaves, short stems, short roots with only few lateral roots and decreased fertility (Ikeda *et al.,* 2006, Marsch-Martine *et al.,* 2006). The proposed targets of *ESR2* include both activators (*CYCD1;1*) and inhibitors of cell cycle (*KRP5*) (Ikeda *et al.,* 2006), whilst *CYCD3;3* is induced and *expansins* are repressed in *35S-ESR2* (Marsch-Martine *et al.,* 2006). We then looked at the expression pattern of *ESR2* as previously determined using promoter-GUS lines (unfortunately, *ESR2* is not represented on the Affymetrix ATH1 array). *ESR2* is expressed during embryogenesis (in apical part at globular stage and in cotyledon primordia at heart stage), in the first two days after germination (in the SAM, RAM and cotyledon tip), in SAM through-out development, very young leaves, in tip and hydatodes of older leaves and young axillary buds (Ikeda *et al.,* 2006, Marsch-Martine *et al.,* 2006; Chandler *et al.,* 2007). Thus, both the proposed targets and the expression pattern of ESR2 also support its regulation of *OBP1*.

5.4 Discussion

5.4.1 OBP1 influence on the cell cycle

The increasing availability of public microarray data has fuelled recent advances in investigating gene function via their digital expression pattern and through their co-expression with other genes of known function. Prominent examples include the identification of proteins involved in secondary cell wall synthesis (Persson *et al.,* 2005) and three glucosyltransferases involved in flavonoid or glucosinolate biosynthesis (Gachon *et al.,* 2005). A similar analysis for OBP1 suggested its potential involvement in cell proliferation. OBP1 is highly expressed in continuously proliferating tissues/organs as well as in cells that re-enter (G0/G1) cell division and its expression is further induced by treatments that strongly affect cell cycle genes. In aphidicolin block/release experiments, in which cultured cells are released from S phase synchronisation, expression of OBP1 drops sharply at the end of the S phase and then gradually increases during G2 and mitotic phase before peaking in the G1 phase (Menges *et al.,* 2003). As expected from its expression pattern among the genes most co-expressed with *OBP1* (ACT, Manfield *et al.,* 2006), the majority can be related to proliferation, including several specific for G1/S transition and replication, e.g. genes encoding for DNA polymerase α, MCM factors and a DNA mismatch repair protein. Together these data may support a role for OBP1 in the phase G1 or during G1/S transition.

Inducible over-expression of *OBP1* in fully developed rosettes resulted in the induction of many cell cycle related genes. In order to identify target genes that may cause these changes we focussed on core cell cycle genes and TFs previously associated with cell cycle progression (Menges *et al.,* 2003; Beemster *et al.,* 2004) and followed their expression after different times following OBP1 activation. In agreement with the proposed role of OBP1 during G1 and G1/S transitions, six out of eight of its early targets (5h after induction) can be associated with G1 or S phase. Two encode for core cell cycle proteins: D-type cyclin, CYCD3;3 and kip-related protein, KRP7. D-type cyclins and kip-related proteins respectively activate and inhibit A-type cyclin dependent kinase (CDKA), and therefore are important regulators of G1-S transition and cell cycle re-entry (Nakagami *et al.,* 1999; Healy *et al.,* 2001; de Veylder *et al.,* 2001 and Nakai *et al.,* 2006). Also induced were two genes, *MCM2* and *MCM5*, encoding for subunits of the mini-chromosome maintenance (MCM) complex that is considered the major DNA helicase in eukaryotic organisms and is essential for replication. Among four induced transcription factors, the expression of two, *AtDOF2;3* and *MYB-like* (At2g40970), was associated with the S-phase in aphidicolin block/release experiments (Menges *et al.,* 2003). In addition, R2R3-type MYB88 TF was shown to restrict divisions late in the Arabidopsis stomatal cell lineage (Lai *et al.,* 2005).

Simultaneous activation of both cell cycle activators and inhibitors is often characteristic for TFs involved in the control of cell proliferation and is believed to provide a feedback mechanism for the tight control of the cell division. Prominent examples are cytokinin regulated NAC with transmembrane motif 1 (NTM1) TF, that controls expression of *KRP2, KRP7* but also *CYCD3;1* (Kim *et al.,* 2006) and AP2 TF ESR2 that early targets include CYCD1;1 and KRP5 (Ikeda *et al.,* 2006). In common with these examples, our data indicate that putative OBP1 targets also include both positive (*CYCD3;3*) and negative regulators (*KRP7* and *MYB88*) of the cell cycle.

Cell division initiated by OBP1 activation progressed, manifested by induction of large subset of mitotic genes measured 10h after DEX application including *CDKB2;1*, *B-type cyclins*, kinesins and M phase associated TFs. This is in agreement with the previous speculations (reviewed by de Veylder and Inze, 2006) on existence of a mechanism by which the activation of DNA replication stimulates mitotic entry. One of the possible scenarios involves engagement of D-type cyclins in regulation of not only G1/S but also G2/M transition. In support of this hypothesis CYCD3;3-associated CDKA was found to be active at both the G1/S and G2/M boundaries (Nakagami *et al*., 2002; Koroleva *et al*., 2004). In common with changes measured 5h after DEX application, OBP1 activation resulted in the induction of both positive (*A, B and D-type cyclins, E2Fa-b, CDKB2;1* and *CDKB2;2*) and negative (*KRP7, WEE1, E2Fc, DEL3*) regulators of the cell cycle (reviewed by de Veylder and Inze, 2007).

These molecular data indicating OBP1 involvement in cell division prompted us to transform MM2d cell cultures with our *IOE-OBP1* construct to investigate OBP1 function. As *OBP1* is G1 specific and targets the expression of genes associated with G1 and G1/S transition we hypothesized that OBP1 activation may affect the duration of the G1 phase. Indeed, OBP1 activation resulted in an increased number of cells within 12h, related to a shortening of the G1phase. Sucrose starvation/re-addition experiments further confirmed OBP1 involvement at the G1/S boundary. Importantly, and in accordance with expression changes measured in *IOE-OBP1* plants, the effect of OBP1 on the G1 duration was perpetuated through the G2 phase, resulting in earlier division. Previous work has demonstrated that effects on the G1/S transition may or may not result in earlier division. For example similar effects on G1/S and G2/M boundaries were observed for tobacco cell cultures over-expressing an active form of *CYCD3;3* (Nakagami *et al.,* 2002), however, ectopic over-expression of *CYCD3;1* also shortens the G1 phase but cells subsequently arrest in the G2 phase (Menges *et al.,* 2006).

Interestingly, only the initial cell division was positively affected by OBP1 activation and subsequently the rate of cell division slowed to below control levels resulting in fewer cells. This subsequent reduction in cell division rate might be related to the induction of negative cell cycle regulators such as *KRP7* and *MYB88* by OBP1. Our data strongly suggest that the normal oscillation of *OBP1* expression during cell cycle is required to maintain a balance between cell cycle activators and inhibitors allowing precise regulation of cell division rate. Importantly, a similar effect on cell division was also found *in planta*: short term activation of OBP1 in fully developed rosettes led to induction of cell proliferation associated marker genes whilst fully developed *35S-OBP1* plants had leaves with fewer cells and less lateral roots suggesting cell cycle repression.

In summary, OBP1 modulates cell cycle activity by affecting the expression of CDKA regulators, S phase specific transcription factors and components of the replication machinery and therefore is important for G1/S transition. By targeting expression of *CYCD3;3* OBP1 may also play a role at the G2/M transition. The oscillation of *OBP1* expression during the cell cycle, in combination with its effect on both cell cycle activators and inhibitors, might confer a feedback mechanism assisting in the tight control of the cell division (Figure 8).

5.4.2 OBP1 influence on cell expansion

Reduced cell number is often compensated for by increased cell size and *vice versa*. This phenomenon was first observed in γ-irradiated wheat seedlings (Harber *et al.,* 1961; Harber, 1962) and together with subsequent studies on a number of mutant and transgenic plants gave rise to the 'compensated cell enlargement theory' (Tsukaya, 2006). A prominant example is *CYCD3;1,* which when over-expressed results in an increased number of significantly smaller cells (Dewitte *et al.,* 2003). As OBP1 affects cell number we also decided to look at the cell size. Consistently between different tissues (root, hypocotyls, epidermis of leaves and cotyledons) and in cell cultures we measured a significant reduction of cell size in response to short- and long-term OBP1 activation. Importantly, both in cell cultures and in *35S-OBP1* plants, this decrease in cell size was independent of the cell number. For example, cotyledon and leaf epidermal cell size were both similarly reduced, however, cell number was increased in cotyledons but decreased in leaves. Therefore, as changes in cell size in response to OBP1 activation can not be explained by the compensation theory, this suggests that OBP1 targets cell growth independent from cell proliferation. To look for possible molecular mechanisms that could explain changes in cell size we checked the expression of genes encoding growth related proteins among transcripts down-regulated in *IOE-OBP1* lines 10h after DEX application. Here, we identified a large number of expansins (eight out of 36) and xyloglucan hydrolases (6 out of 33). It is believed that primary wall-loosening factors such as expansins directly induce turgor-driven wall extension, whereas secondary wall-loosening factors (such as XTHs) modify the structure of the wall, rendering it more responsive to primary wall-loosening events and therefore both are important for vacuolar cell growth (Li *et al*, 2003). Therfore, OBP1 affects growth independently from its effects on cell proliferation and it may do so by targeting the expression of genes encoding for cell wall loosening enzymes (Figure 8).

5.4.3 OBP1 molecular interactions and their biological significance

In order to gain better insight into the biological function of OBP1 we needed to be certain on the direct nature of OBP1 regulation of the observed gene expression changes. We were primarily interested whether the early target genes identified in our Q-RTPCR screen are also direct OBP1 target genes. Therefore, from the subset of eight transcripts induced within 5h of OBP1 activation, we selected two for which expression was also reduced in *RNAi-OBP1* lines. Subsequently, using ChIP/Q-PCR we proved that induction of *CYCD3;3* and *AtDOF2;3* was caused by direct binding of OBP1 to their promoter sequences.

Previously, *in vitro* evidence indicated that the *ocs* element in the *GST6* promoter was bound by OBP1 (Zhang *et al.,* 1995, Chen *et al.,* 1996). As we demonstrated that 5h was sufficient for both binding to the promoter and regulating the expression of OBP1 direct targets, we also performed these experiments for *GST6*. However, its expression was not changed and there was no enrichment of the promoter fragment containing the *ocs* element. Importantly, *in vivo* proof for *GST6* regulation is also lacking for the other OBPs, OBP2 and OBP3 (Kang *et al.,* 2003; Ward *et al.,* 2005; Skirycz *et al.,* 2006). This highlights the limitations of *in vitro* based studies in distinguishing between putative and functional interactions.

Our searches of literature and available expression data revealed *OBP1* among 51 direct targets of the AP2/ERF TF ESR2 (Figure 8) (Ikeda *et al.,* 2006). The position of ESR2 as an upstream regulator of OBP1 is supported by the presence of an ESR2 binding site in the promoter of *OBP1* and this element is conserved in the corresponding promoter region of *OBP1* orthologs in poplar and *Brassica oleracea*. Furthermore, similarities in the tissue specific expression pattern and the 35S phenotypes strengthen the connection between OBP1 and ESR2. This was particularly interesting as ESR2, similarly to OBP1, is associated with cell proliferation (Ikeda *et al.,* 2006; Marsch-Martinez *et al.,* 2006; Chandler *et al.,* 2007).

To establish the biological context in which OBP1 may regulate the cell cycle we looked for overlap in expression patterns between *OBP1*, its early targets and *ESR2***.** As D-type cyclins act as sensors that integrate developmental and environmental signals with the cell cycle machinery, we were particularly interested in processes that involve cell cycle activation or re-entry. Considering *OBP1*, *ESR2* and D-type cyclin expression in the root apex of germinating seedlings and recent publication implicating cell cycle activation in radicle protrusion (Masubelele *et al.,* 2006) we hypothesized that OBP1 may be involved in the activation of cell division in the RAM during seed germination. Consistent with this, the expression of OBP1 early target genes (*CYCD3;3, AtDOF2;3, MCM2/5* and *MYB-like* TF) follows *OBP1* expression increases shortly after imbibition. As expression of *CYCD3;3* was decreased in *RNAi-OBP1* lines and knock-outs of D-type cyclins were characterised by delayed germination (Masubelele *et al.,* 2006) we scored timing of radicle protrusion. Initially, the comparison of seeds of homozygous *RNAi-OBP1* and empty control lines indicated that germination could be affected. As maternal or seed batch effects can be important factors affecting germination we decided to measure radicle protrusion using a segregating *RNAi-OBP1* seed population. This revealed that germination was unaffected in our *RNAi-OBP1* lines. However, as there is only partial silencing of *OBP1* and still considerably high *CYCD3;3* transcript levels in investigated RNAi lines, OBP1 may still have an undetermined role in radicle protrusion.

Another possibility is the involvement of OBP1 in the activation of cell division during early cotyledon development. It has been recently shown that ESR2 and its homolog ESR1 pay a role in both cotyledon initiation and boundary maintenance (Chandler *et al.,* 2007). Whilst the later of these has been shown to be dependent on ESR2 mediated activation of *CUP-SHAPED COTYLEDON 1* (CUC1) (Ikeda *et al.,* 2006), the molecular mechanism of ESR2 regulation of cotyledon initiation remains to be elucidated. Considering the involvement of OBP1 in the cell cycle and that, similarly to ESR2, *OBP1* expression is associated with cotyledon emergence during embryogenesis it seems possible that OBP1 may be involved in the ESR2-mediation of cotyledon initiation. In support of a role in cotyledon development, and in contrast to the situation in true leaves, cotyledons of *35S-OBP1* plants were characterised by increased cell number.

In summary, OBP1 directly regulates the expression of *CYCD3;3* and TF *AtDOF2;3* whilst itself being regulated by ESR2. As expected by its effect on D-type cyclin, OBP1 seems most likely to be involved in cell cycle activation and re-entry in a developmentally specific manner.

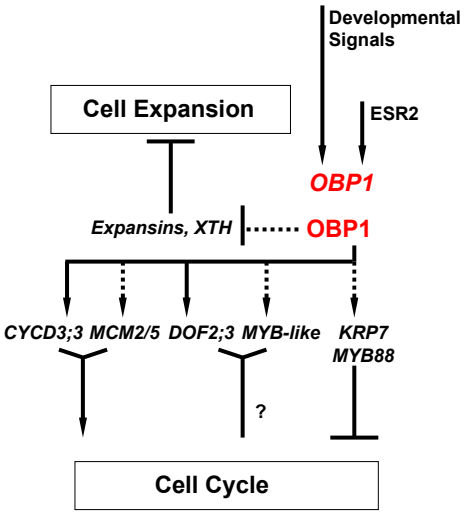


Figure 8. Model of OBP1 regulatory network. OBP1 activates expression of cell cycle activators and repressors; *CYCD3;3* and *AtDOF2;3* were proven to be direct OBP1 targets. In contrast, OBP1 negatively affects cell expansion. In addition *OBP1* expression is regulated by TF ESR2.

5.5 Material and Methods

5.5.1 General methods

Standard molecular techniques were performed as described (Sambrook *et al*., 2001). Oligonucleotides were obtained from MWG (Ebersberg, Germany). DNA sequencing was performed by AGOWA (Berlin, Germany). Unless indicated, other chemicals were purchased from Roche (Mannheim, Germany), Merck (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany) or Sigma (Deisenhofen, Germany). For sequence analyses the tools provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), MIPS (http://mips.gsf.de/), The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/), and the Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de) were used.

5.5.2 Plant material

If not indicated differently Arabidopsis seeds were grown in a growth chamber with a 16-h day length provided by fluorescent light at 80 or 120 µmol m^{-2} s^{-1} and a day/night temperature of 20/16°C and relative humidity of 60/75%. Short day conditions were as follow: 8-h day length provided by fluorescent light at 150 µmol m^{-2} s^{-1} and a day/night temperature of 20/18°C and relative humidity of 60/75%. With the exception of pJawohl8-RNAi vector that requires strain GV3101RK(pMP90) we used *Agrobacterium tumefaciens* strain GV3101 (pMP90) to transform *Arabidopsis thaliana* (L.) Heynh. cv. C24 (Clough, 2005). In tissue culture, seedlings were grown in half-strength Murashige and Skoog medium (0.5 MS) supplemented with 1% sucrose and solidified with 0.7% agar under a 16-h day (140 µmol m-2 s-1) / 8-h night regime (22°C). For root experiments plants were grown vertically under the same conditions except 1% agar was used. If not indicated differently, measurements were performed on fully developed rosettes harvested from randomized 5- to 6-week-old plants prior to bolting. For induction experiments plants were sprayed with 25µM DEX solution or grown in media containing 5 µM DEX.

5.5.3. Cell cultures

MM2d cell cultures were kindly provided by Prof. James Murray (University of Cambridge, UK). Cultures were maintained by weekly subculturing of 3.5 ml saturated culture into 100 ml fresh MSS media (Murashige and Skoog medium supplemented with 3% w/v sucrose, 0.5 mg/l NAA and 0.05 mg/l kinetin, with pH adjusted to 5.8 using 1N KOH) in 300 ml narrow-necked Erlenmeyer flasks and grown in continuous darkness with rotation at 130 rev/min at a temperature of 26°C. For DEX experiments cells were induced with 5µM DEX or treated with

0.005% EtOH (mock). In time series experiments samples were taken at given time points from one flask. To minimize exposure to light all harvests were conducted in darkness with the help of green light torch. For growth analysis cell clumps were eliminated by 30min incubation with equal volume of 1% cellulase and 0.6% macerase in 0.6M sorbitol at 37°C. Subsequently cells were microscopically observed and counted using a counting chamber and Olympus BX51 microscope. Cell size was analyzed using cell^P Software (Olympus). Sucrose starvation was performed as described by Menges *et al.* (2002). Transformation of MM2d cells was performed as described by Menges *et al.* (2004) with *OBP1-GUS* (pGPTV-KAN) and *IOE-OBP1* (pBI-GR-KAN) constructs.

5.5.4 Constructs

35S-OBP1: Polymerase chain reaction (PCR) was used to amplify the *OBP1* coding region using Arabidopsis C24 leaf cDNA as template. Primer sequences were as follows: *OBP1*-fwd., 5´-GTTTAAACATGAAACCTAACGGCGTAACAG-3´ (added *PmeI* restriction site underlined); *OBP1*-rev., 5´-TTAATTAATTATTTGAGTTTTCCCGGAG-3´ (added *PacI* restriction site underlined). The *OBP1* cDNA was inserted into pUni/V5-His-TOPO and, after sequence confirmation, cloned via the *PmeI/PacI* sites into a modified pGreen0229 plant transformation vector (www.pgreen.ac.uk) containing a Cauliflower Mosaic Virus (CaMV) 35S promoter (Skirycz *et al*., 2006). *RNAi-OBP1:* A 3'-UTR *OBP1* fragment was PCR amplified from a leaf cDNA library using following oligonucleotides; RNAi-fwd., 5´-CACCTTTTCAACTGTTGGTACTGCGACAACG-3´ and RNAi-rev., 5´-GACACAAACTAAGAGATCAACATCACC-3'. The PCR product was cloned into pENTR/D-TOPO of the Gateway System. After sequencing, the *OBP1* fragment was transferred to the destination vector pJawohl8-RNAi (kindly provided by Dr. Imre Somssich, MPI for Plant Breeding, Cologne) or pBin19-RNAi (kindly provided by Dr. Alisdair Fernie, MPI of Molecular Plant Physiology, Golm). *Promoter-GUS fusion*: A ~2kb 5´ genomic fragment upstream of the ATG start codon was amplified by PCR using primers *OBP1*GUS-fwd., (5´GTCGACGGAACAAATATGATCACAATGATAGATCATA-3´) and *OBP1*GUS-rev., (5´-GCTCTAGAAATCGGAGAAAGGTTGAAGCTTTAAGG-3´) from Arabidopsis C24 genomic DNA. The promoter fragment was inserted into plasmid pCR-Blunt II-TOPO and verified by sequencing. Subsequently, the promoter was fused to the *E. coli* β-glucuronidase (GUS) reporter gene in pGPTV-Kan (Becker *et al*., 1992), previously cut with *SalI* and *XbaI*, resulting in plasmid *OBP1-GUS*. IOE-OBP1: Primer sequences were as follows: OBP1-fwd., 5´-TCTAGAATGCCGACGTCTGACTCCGGTG-3´ (added *XbaI* restriction site underlined); OBP1-rev., 5´-TCTAGACATTTGAGTGAGTTTCTCGGAGTTG-3´ (added *XbaI* restriction site

underlined). The *OBP1* cDNA was inserted into TOPO-TA and, after sequence confirmation, cloned via the *XbaI* sites into a d143 (pBI-GR) vector (Lloyd *et al.,* 1994).

5.5.5 Quantitative real-time PCR

Total RNA was isolated using TRIzol reagent as described in the manual. To generate first-strand cDNA RNA (2µg) was digested with RNase free DNaseI (Sigma, Deisenhofen, Germany) and reverse transcribed with Superscript™ III reverse transcriptase (Invitrogen, Karlsruhe, Germany) in a reaction volume of 20 µl. cDNA quality was assessed with primers designed against 3' and 5' ends of *GAPDH* (AT1G13440). Quantitative RT-PCR was performed with SYBR Green reagent (Applied Biosystems Applera, Darmstadt, Germany) in 96 (10µl reaction) or 394 well-plates (5 µl reaction) on a GeneAmp 7300 Sequence Detection System or on an ABI PRISM 7900 HT sequence detection system (Applied Biosystems), respectively. Primer sequences are given in the supplementary material. Data were normalized to *ACTIN2* (AT3G18780), *UBIQUITIN10* (AT4G05320), *GAPDH* (AT1G13440), *PDF2* (AT1G13320) and expressed protein (AT2G32170); ΔCt = Ct (gene) – Ct (mean (house-keeping genes)). Ct refers to the number of cycles at which SYBR Green fluorescence in a PCR reaches an arbitrary value during the exponential phase of DNA amplification, set at 0.2 in all experiments.

5.5.6 Microarray experiments

Whole rosettes pooled from three plants were harvested. Extraction, labeling, hybridization, and scanning were done as described (Redman *et al.,* 2004). Each sample was hybridized to a single Affymetrix ATH1 Genome array (ATH1) at the German Resource Center for Genome Research, Berlin (www.rzpd.de). Data normalization has been previously described (Hannah *et al.,* 2005).

5.5.7 Chromatin immuno-precipitation

Plant material (approximately 400mg) was processed as described by Leibfried *et al.* (2005). Polyclonal anti-GR antibody (Abcam, Cambridge, UK) was used at concentration 1µg per sample. Obtained DNA samples were measured in 10µl reaction with SYBR Green reagent (Applied Biosystems) in 96 well-plates on a GeneAmp 7300 Sequence Detection System (Applied Biosystems). Primer sequences are given in the supplementary material. Data were normalized to genomic fragment of UBIQUITIN10 (AT4G05320), ΔCt = Ct (promoter fragment) – Ct (UBQ fragment). Ct refers to the number of cycles at which SYBR Green fluorescence in a

PCR reaches an arbitrary value during the exponential phase of DNA amplification, set at 0.2 in all experiments.

5.5.8 Electrophoretic-mobility shift assay (EMSA)

OBP1 protein was purified from *E.coli* strain SCS1/pSE111 carrying lacIQ and conferring kanamycin resistance. Expression vector was kindly provided by Dr. Birgit Kersten (Kersten *et al.,* 2003). Protein expression was induced using 1mM IPTG and cells were harvested 3h after induction. Protein purification was performed under native conditions using Ni^{+2}-agarose according to Qiagen protocol (Qiagen, Hilden, Germany). Purified proteins were separated on a 12.5% polyacrylamide gel and protein concentrations were determined by Bradford assay (Bradford, 1976). RD-800 labeled DNA sequence (5'-AAATAATCATAAAGTATTAAAGTAATATATAAC-3'), its complementary strand as well as competitor fragments (5'-AAATAATCATAAAATATTAAAGTAATATATAAC-3' and 5'-AAATAATCATAAAATATTAAAATAATATATAAC-3') were ordered from MWG (Ebersberg, Germany). Annealing was performed by heating the primers to 100°C followed by slow cooling to room temperature (RT). Binding reaction was performed at RT for 20min in 10µl volume ((2µl 5x binding buffer [100mM Hepes, pH 7.6, 5mM EDTA, 50mM $(NH4)_2S0_4$, 25mM DTT, Tween 20, 1 % (w/v), 150 mM KCl], 1µl 0.1mM labeled DNA fragment, 1µl of BSA (10mg/ml), 2µl poly d(I-C) (10units per ml, 0.35µg/µl)), approximately 70ng of OBP1 protein and 10 or 100x molar excess of unlabelled competitor sequence. DNA-protein complexes were separated on 6% DNA retardation gels (Invitrogen) whilst RD-800 signal was detected using the Odyssey Infrared Imaging System from LI-COR Biosciences.

5.5.9 GUS assays

β-Glucuronidase activity was determined histochemically as described (Plesch *et al.*, 2001) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as substrate (Jefferson *et al.*, 1987). Pictures were taken using stereomicroscope MZ 12.5 (Leica) or BX51 light microscope (Olympus). Embryos were visualized as described in Chandler *et al.* (2007).

5.5.10 Microscopy

Epidermis cell sizes were analyzed following chlorophyll removal with 100% ethanol using BX51 light microscope and cell^P Software (Olympus).

5.5.11 Flow Cytometry

Frozen cell culture samples were processed using Cystain UV Precise P kit (Partec, Münster, Germany). Subsequently nuclei were analyzed with the CyFlow flow cytometer with the FloMax Software (Partec, Münster, Germany) and the G1/S/G2 distribution was estimated using DNA cell cycle analysis software Multicycle AV for Windows (Phoenix Flowsystems, San Diego, CA, USA).

4.5.12 AGI code numbers

The AGI code numbers for the genes tested in this study are as follows: *Actin2* (AT3G18780), *UBQ10* (AT4G05320), *GAPDH* (AT1G13440), *PDF2* (AT1G13320), *EXPRESSSED* (AT2G32170), *MCM2* (AT1G44900), SNC1 (AT4G02150), *KINESIN1* (AT5G33300), *KINESIN2* (AT3G44050), *EXPRESSED* (AT5G17160), *CDKB2;1* (AT1G76540), *CKS2* (AT2G27970), *CYCA2;2* (AT5G11300), *CYCB1;3* (AT3G11520), *CYCB2;4* (AT1G76310), *CYCD3;2* (AT5G67260), *CYCD3;3* (AT3G50070), *CYCD4;1* (AT5G65420), *DEL3* (AT3G01330), *E2Fa* (AT2G36010), *MCM5* (AT2G07690), *KRP7* (AT1G49620), *TAZ* (AT5G67480), *MYB-like* (AT2G40970), *OBP1* (AT3G50410), *DOF2;3* (AT2G34140), *MYB88* (AT2G02820), *GST6* (AT2G47730).

5.6 Acknowledgements

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6. General discussion and outlook

6.1 Overview of the three DOF TFs

This PhD thesis concentrated on the functional characterisation of the DOF TFs OBP1, OBP2 and AtDOF4;2 that were selected in a larger screen for TFs conferring distinct growth phenotypes when constitutively over-expressed. Over-expression of *OBP1* and *OBP2* severely reduced growth whilst over-expression of *AtDOF4;2* affected branching.

To identify biological processes regulated by OBP1, OBP2 and AtDOF4;2, molecular and physiological analyses of transgenic gain- and loss-of-function lines (35S, RNAi, IOE) using both targeted and profiling approaches were performed. In addition, the expression pattern of the studied TFs and selected target genes was investigated using promoter-GUS lines and by mining publicly available microarray data. Finally, for selected candidate target genes, TF binding to the promoters was verified by ChIP/Q-PCR and EMSA.

This combinatorial approach revealed that OBP2 is involved in the control of indole glucosinolate / auxin homeostasis by regulating the enzyme at the branch of these pathways, CYP83B1 (Skirycz *et al.,* 2006). OBP1 was shown to control entry into the cell cycle by affecting the D-type cyclin/RB signaling pathway most likely at specific developmental stages. In agreement with the divergent expression pattern of both genes as well as their low homology, these data indicate that *OBP1* and *OBP2* are likely to have distinct functions. In contrast, OBP1, OBP2 and another DOF TF, OBP3, were previously suggested to share a common role in salicylic acid and auxin signalling (Kang and Singh, 2000). This was mostly based on *in vitro* evidence showing their interaction with the bZIP TF OBF4 on the promoter of the *GST6* gene (Chen *et al.,* 1996; Kang and Singh, 2000). This PhD work, as well as functional analysis of OBP3, have failed to provide *in vivo* support for the *GST6* regulation (Ward *et al.,* 2005; Skirycz *et al.,* 2006). These data highlight the potential limitations of *in vitro* based studies in distinguishing between putative and functional interactions.

In contrast to OBP1 and OBP2, no previous characterization of AtDOF4;2 has been reported. We found that under certain stress conditions AtDOF4;2 negatively regulates flavonoid biosynthetic genes whilst in certain tissues activates hydroxycinamic acid production. As such, it is a good example of a TF whose function depends on the biological context. As DOF TFs have been previously shown to interact with other TFs (reviewed by Yanagisawa, 2004), we hypothesized that this dual function is most likely related to specific interactions with other transcription factors or proteins (Skirycz *et al.,* 2007). Interactions between TFs are not limited to DOF TFs and are a common mechanism to confer context-dependent regulation. For example,

the bHLH TFs ENHANCER OF GLABRA3 and GLABRA3, interact with different MYB TFs in the regulation of either seed mucilage production, tannin biosynthesis or trichome initiation (reviewed by Broun, 2005).

6.2 OBP1, OBP2 and AtDOF4;2 within the context of the DOF TF family

6.2.1 Functional diversity of DOF TFs

Taking together the data presented here and elsewhere it is clear that DOF TFs are involved in divergent plant specific processes including seed development and germination (Vicente-Carbajosa *et al.,* 1997; Washio *et al*., 2001; Gualberti *et al.,* 2002; Mena *et al.,* 2002; Papi *et al.,* 2002; Isabel-LaMoneda *et al.,* 2003; Diaz *et al.,* 2005; Moreno-Risueno *et al.,* 2007), primary and secondary metabolism (Yanagisawa and Sheen, 1998; Yanagisawa, 2000; Skirycz *et al.,* 2006, 2007), flowering (Imaizumi *et al.,* 2005) and light signaling (Park *et al.,* 2003; Ward *et al.,* 2005).

It is interesting what characteristics might confer such functional diversity. One of the most likely contributing factors is their divergent expression patterns which can vary greatly during development and under different environmental conditions. As discussed in the previous section, the biological context may greatly influence TF function (Skirycz *et al.,* 2007) and this may relate to the presence or absence of certain co-factors under certain conditions. This is particularly likely given the known combinatorial nature of eukarotic transcriptional regulation with specific protein-protein interactions at the promoters of target genes shown to be crucial for TF specificity. At the protein level, the recognition of specific protein partners may be facilitated by the divergent protein structure outside of the conserved DOF domain. For example both N- and C-terminal regions outside the DOF domain were found to be important for specific binding of HvDOF17 and HvDOF19 to HvGAMYB TF in barley endosperm (Moreno-Risueno *et al.,* 2007). In addition to taking alternative protein-partners, some DOF TFs are able to self associate, at least *in vitro*, whilst others, such as HvDOF17, do not (Yanagisawa, 1997; Moreno-Risueno *et al.,* 2007) and this could contribute to *cis*-element recognition. In addition to divergence in protein-protein interactions, another contribution to the functional diversity of DOF TFs may come from differences in their activation capacity. For example, in maize DOF1 and DOF2 compete for the same binding sites *in vivo,* however whilst DOF1 activates target gene expression, DOF2 acts as a repressor due to it lacking a functional activation domain (Yanagisawa and Sheen, 1998). Again, such characteristics are not specific to the DOF family, with a well-described example being the competition between the transcriptional activators E2Fa

and E2Fb and the E2Fc repressor which is devoid of an activation domain (reviewed by Inze and De Veylder, 2006).

In contrast to such specific protein-protein interactions there is little evidence for differences in *cis*-element recognition among different DOF TFs. In this respect, importantly, it was demonstrated that DNA sequence flanking the DOF binding site has no effect on binding efficiency (Yanagisawa and Schmidt, 2001). Despite this, and in line with the possibility for sef-association discussed above, a number of characterised DOF TFs show preference towards multiple DOF sites. For example the promoter region of *KST1* bound by StDOF1 contains three (two in tandem) DOF elements and all three are required for full *KST1* activity (Plesch *et al.,* 2001). Similarly, promoter fragments of *CYCD3;3* and *AtDOF2;3* bound by OBP1 are characterized by the presence of tandem DOF sites. As experimental evidence is currently missing we can only speculate that binding to multiple DOF elements is related to the ability of certain DOF TFs to self-associate or interact with other DOF TFs.

Promoter bashing experiments are planned to establish whether tandem DOF elements found in *CYCD3;3* and *AtDOF2;3* promoters are functional, whilst protein-protein interactions will be examined using immuno-precipitation and yeast two-hybrid assays.

6.2.2 Evolution of the DOF TF family

When viewed from an evolutionary rather than molecular perspective, the divergent functions of DOF TFs discussed above are related to the duplication-led expansion of the DOF family (Moreno-Risueno *et al.,* 2006; Yang *et al.,* 2006). Duplicated genes undergo different fates and thus can retain or lose their original function (retention and pseudogenisation, respectively), gain new function (neofunctionalization) or partition the function of the ancestral gene (subfunctionalization). Moreover they can be also affected by epigenetic changes. Recently, these different evolutionary fates were revealed for pairs of paralogous DOF genes identified within poplar, Arabidopsis and rice by using a maximum-likelihood phylogenetic tree constructed from full-length protein sequences (Yang *et al.,* 2006).

Although evolutionally analyses provide insight into the genetic diversification of the DOF TFs, they also demonstrate that TF function may be conserved across the plant kingdom. As mentioned in the Introduction, based on the phylogenetic tree of DOF domains from the unicellular alga *Chlamydomonas reinhardtii*, the moss *Physcomitrella patens* and the angiosperms Arabidopsis and rice, DOF TFs were separated into three groups (Shigayo *et al*., 2006) (Figure 1). Group A is the most ancient and was suggested to be associated with the biological processes common among all green organisms. Consistent with this assumption four

(CDF1-3 and COG1) among seven Arabidopsis genes belonging to this group was previously described in connection to light signaling (Park *et al*., 2003; Imaizumi *et al*., 2005). Group B is moss specific whilst group C, suggested to be related to organ development in vascular plants, contains the majority of the angiosperm DOF TFs including OBP1, OBP2 and AtDOF4;2. Indeed, a number of TFs belonging to group C can be related to seed development and germination, including HvPBF, HvSAD, AtDAG1/2 and OsDOF3 (Vicente-Carbajosa *et al.,* 1997; Washio *et al*., 2001; Gualberti *et al.,* 2002; Mena et al., 2002; Papi *et al.,* 2002; Isabel-LaMoneda *et al.,* 2003; Diaz *et al.,* 2005). Additionally we gained evidence on a role of OBP1 during embryogenesis and radicle protrusion whilst *AtDOF4;2* is expressed in testa of seeds. Expression of a relatively high number of C-type DOF TFs was also associated with vasculature including *OBP2, OBP3, DAG1, AtDOF2;4* and *AtDOF5;8* (Papi *et al.,* 2002; Ward *et al.,* 2005; Skirycz *et al.,* 2006; Konishi and Yanagisawa, 2007). However, despite several examples to support such evolutionary functional grouping, limitations and ambiguity are also apparent. For example *CDF1*, a member of the A subfamily, is light responsive but is also specifically expressed in the vasculature (Imaizumi *et al.,* 2005) whilst OBP3, belonging to group C, is involved in the regulation of light signaling (Ward *et al.,* 2005). Of the three TFs we characterized, *AtDOF4;2* is notable with respect to the validity of these evolutionary groupings as it was expressed in organs specific for angiosperms (e.g. seeds and anthers) but regulates phenylpropanoid metabolism which is universal to the plant kingdom (Skirycz *et al.,* 2007). Thus, elucidating the function of further DOF TFs is necessary to further evaluate the relevance of the evolutionary relationships among members the DOF TF family.

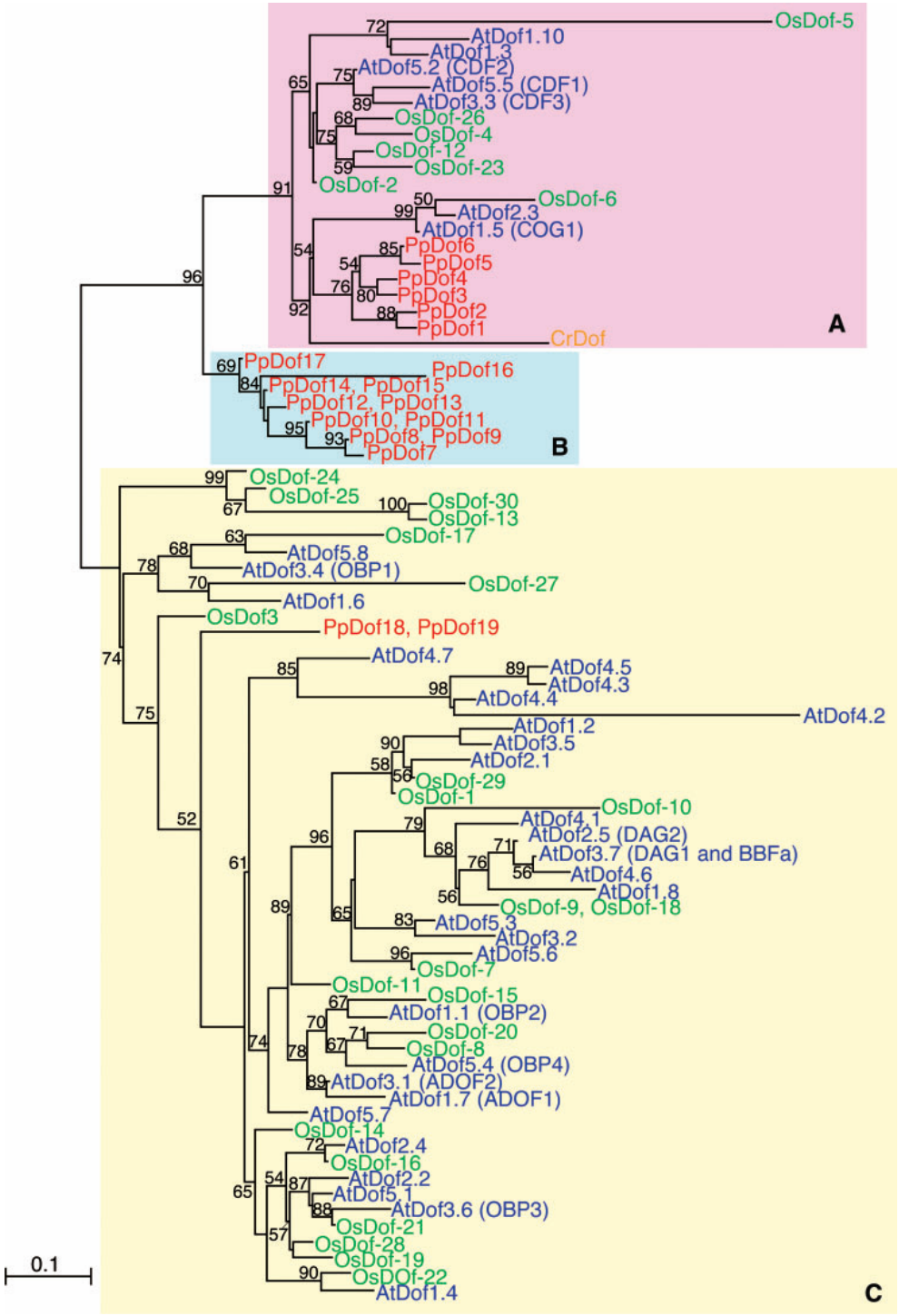

Figure 1. A phylogenetic tree generated of the DOF domains from Arabidopsis, rice, moss and Chlamydomonas. Reproduced from Shigayo *et al.* (2007).

6.2.3 Homologs of *OBP1*, *OBP2* and *AtDOF4;2*

In the best cases, phylogenetic sequence comparisons can assist in revealing gene function. For example, in all so far studied monocots, orthologs of the barley PBF DOF TF were found to have a conserved function during germination (Vicente-Carbajosa *et al.,* 1997, Washio *et al*., 2001; Mena *et al*., 2002). Similarly, paralogous genes, such as *DAG1* and *DAG2*, both affecting germination in Arabidopsis, can be functionally related (Gualberti *et al.,* 2002; Papi *et al.,* 2002). For this reason, we investigated paralogs and orthologs of *OBP1*, *OBP2* and *AtDOF4;2*. All three TFs have paralogous genes in Arabidopsis and were included in the study of duplicated gene function in the DOF family by Yang et *al.* (2006). At the protein level both AtDOF4;2 and OBP1, and their respective paralogs (AtDOF4;4 and AtDOF5;6) retained motif structure of the ancestral protein suggesting their function may be conserved (Fig. 2a-2b). In the case of *AtDOF4;2* and *AtDOF4;4*, which are tandem duplicates, the evidence for functional redundancy is stronger as they also have identical expression patterns as determined by using promoter-GUS lines (Fig. 2d-2g). In contrast, the expression patterns of *OBP1* and *AtDOF5;6* has diverged during evolution; whilst *AtDOF5;6* expression is restricted to shoot apical meristem, *OBP1* is also highly expressed in other proliferating organs such as callus, cell cultures and nodes (Fig. 2h). It would be interesting to investigate whether similarly to OBP1, AtDOF5;6 is also involved in cell cycle regulation. In the case of *OBP2*, both it and its paralog (AtDOF2;2) are phloem specific (Fig. 2h), however, during evolution OBP2 has lost several of the ancestral motifs which would imply functional divergence (Fig. 2c).

In addition to paralogs we also looked at orthologs of *OBP1*, *OBP2* and *AtDOF4;2*. Surprisingly as phenylpropanoid metabolism is universal among the plant kingdom, no orthologs were found for *AtDOF4;2* or its paralog *AtDOF4;4* in any of the sequenced plant species. This was also the case for *OBP2*, but in this case it was expected as glucosinolate biosynthesis is restricted to plants in the order *Capparales*. In contrast, *OBP1* orthologs could be identified in several dicot species including poplar, grape and potato (section 2.2, Table 1). This is in agreement with our hypothesized role for OBP1 during cotyledon initiation. Significantly, the available data for poplar indicate the ortholog of *OBP1* is also expressed in the cambial and shoot apical meristem, supporting a potentially conserved function (Fig. 1i-1k). To verify such proposed evolutionary scenarios it would be interesting to functionally characterize OBP1, *OBP2* and *AtDOF4;2* paralogs as well as the *OBP1* ortholog in poplar .

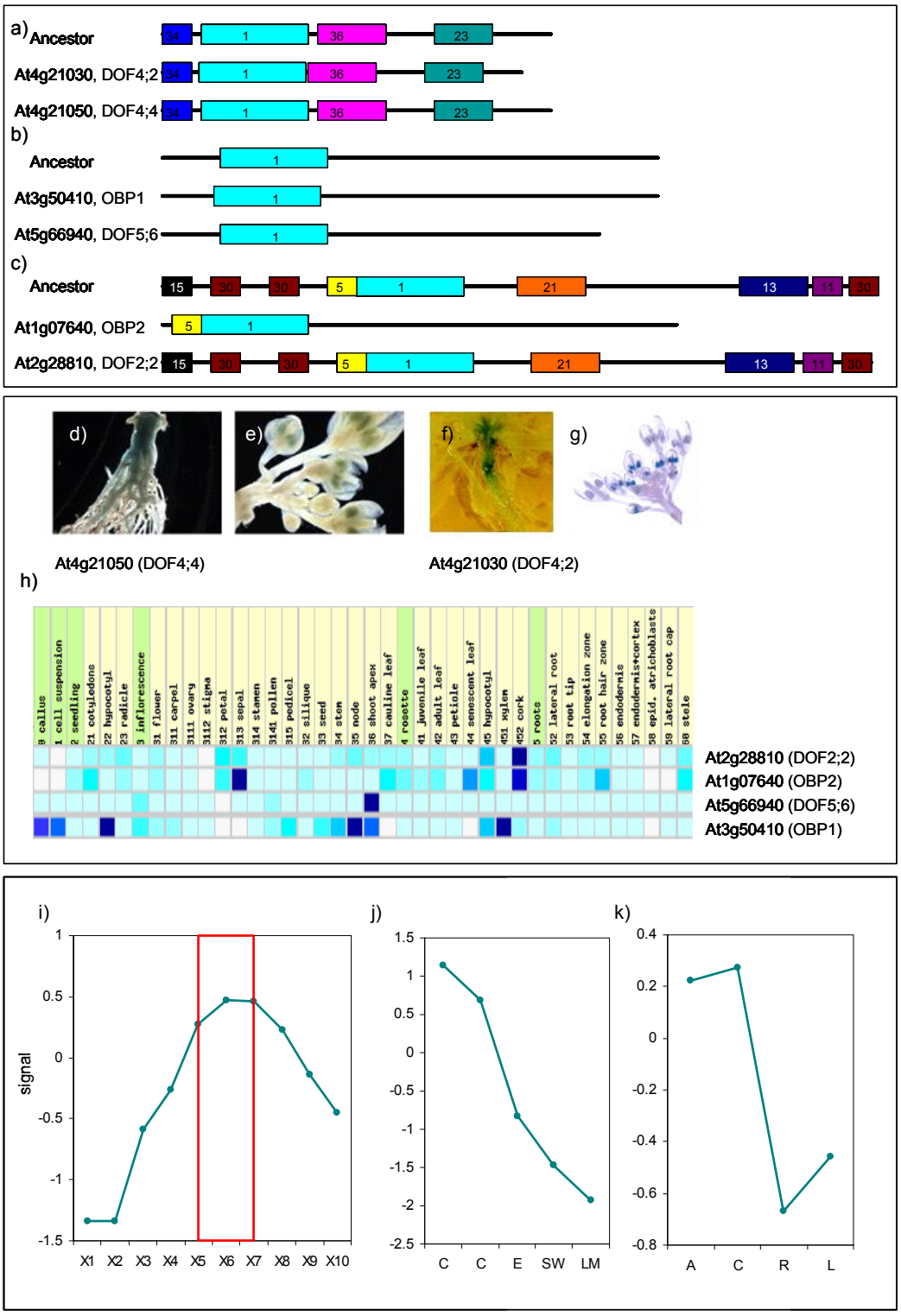

Figure 2. Motif structures and expression patterns of *OBP1*, *OBP2*, *AtDOF4;2* and their paralogs. (a-c) Motif structure of gene duplicates and their ancestors. Boxes labeled with numbers represents coding regions of protein motifs. Reproduced from Yang *et al.* (2006). (d-g) Expression pattern of *AtDOF4;2* (At4g21030) and *AtDOF4;4* (At4g21050) was assessed using promoter-GUS lines. Photos

depict root (d, f) and flower buds (e, g). *AtDOF4;4* GUS lines were prepared by Dr. Isabell Witt. (h) Expression of *OBP1* (At3g50410) and *AtDOF5;6* (At5g66940), *OBP2* (At1g07640) and *AtDOF2;2* (At2g28810) in different plant organs. Data are from the AtGenExpress developmental series and were represented using MetaAnalyzer at the Genevestigator website (Zimmermann *et al.,* 2004; Schmid *et al.,* 2005) (i-j) Expression of poplar *OBP1* ortholog across wood forming meristems. X1 – X10 – sections across wood forming meristem. Data are from two independent experiments (Schrader *et al.,* 2004). The frame indicates cambial cells. C – cambium, E – expansion zone, SW – secondary wall formation zone, LM – late maturation zone. (k) Expression of *OBP1* ortholog in different poplar meristems (A – apical, C – cambial, R – root) and in leaves (L) (Schrader *et al.,* 2004).

6.3 Transcription factors

Transcriptional regulation in general and transcription factors in particular are crucial for the development and environmental responses of all eukaryotic organisms and therefore the characterization of transcriptional regulatory networks has attracted much attention. Importantly, their key role also makes them attractive targets for applied research. For instance the utility of transcription factors for plant biotechnology has been emphasized in recent years. Examples of such approaches include the over-expression of maize TF DOF1 in Arabidopsis and potato leading to improved nitrate assimilation under nitrate deficiency (Yanagisawa *et al.,* 2004) or the use of CBF/DREB TFs to enhance stress tolerance (Kasuga *et al.,* 1999).

Given such key importance, it is not surprising that the toolbox for the study of transcriptional regulation has grown large over the years. However, despite the multitude of *in vitro*, *in vivo* and *in silico* methods all can be assessed as having both advantages but also certain limitations. For example, in the past identification of target genes usually relied on expression profiling of constitutive gain-of-function mutants, requiring a careful separation of primary and secondary effects of TF over-expression (reviewed by Zhang, 2003). Severe pleiotropic phenotypes, similar to those we observed for OBP1 and OBP2, have been often reported. An excellent example are the *CBF1-3* TFs, which when over-expressed result in their supposed primary function - increased freezing tolerance, but also dwarfish stature and delayed development (Gilmour *et al.,* 2000). Inducible over-expression overcomes some of the problems connected to constitutive activation allowing easier separation of primary and secondary effects, however, it may still lead to artifacts related to the loss of TF specificity. In contrast, ChIP is widely regarded as the ‘gold standard’ for TF target identification, allowing the direct identification of target genes. However, not only is it a demanding technique, TF binding has been shown to be highly condition-dependent leading to moderate levels of both false positives and negatives (reviewed by Blais and Dynlacht, 2005). Furthermore, due to the considerable effort and resources required,

comprehensive ChIP-on-CHIP data are lacking for the higher eukaryotes and, as mentioned previously, there are only two published studies using ChIP-on-CHIP in plants (Lee *et al.,* 2007; Thibaud-Nissen *et al.,* 2006).

Therefore, considering such benefits and limitations of currently available methods, a combination of different approaches is usually needed for the robust functional characterization of a given transcription factor. For example accuracy of target gene prediction can be improved by combining ChIP data, expression profiling of transgenic lines, promoter analysis and co-expression analysis. A good example of this comes from recent work in yeast, which has the most comprehensive data, in which unknown TF-binding interactions were predicted from combining eight different forms of evidence including ChIP-on-CHIP data and co-expression (Beyer *et al.,* 2006). In plants such comprehensive data is available only for specific TFs, for example a combinatorial approach was used to discover target genes of MADS box TF AGAMOUS during carpel and stamen formation. Expression profiling of inducible AGAMOUS lines was followed by promoter analysis, ChIP, EMSA and co-expression analysis (Gómez-Mena *et al.,* 2005). We employed a similar approach for the identification of OBP1 target genes and in the future this will be extended to OBP2 and AtDOF4;2. Moreover protein partners of OBP1, OBP2 and AtDOF4;2 will be identified using immuno-precipitation and yeast two-hybrid assays, providing an additional form of evidence.

Assuming a similar progression to recent years, in the future existing methods for studying transcriptional regulation will be improved and new technologies will emerge. A promising example is that of TF 'calling cards' which offers a possibility for *in vivo* identification of TF target genes on the whole genome scale and is an alternative to ChIP (Wang *et al.,* 2007). Briefly, this method relies on directed integration of a retrotransposon, labeled with a unique short DNA sequence (referred to as 'calling card' or 'barcode'), close to the binding site of a studied TF. Subsequently DNA is purified and analyzed for the presence of 'barcode' sequences. Importantly this method completely eliminates the need of formaldehyde fixation and immuno-precipitation. Moreover, in contrast to ChIP, it relies on detecting the 'barcode' rather than quantifying the enrichment which simplifies data analysis and interpretation. Finally cells can be transformed with more than one unique TF-retrotransposon combination potentially allowing simultaneous detection of binding sites for many TFs. For the moment 'calling card' technology is only available for yeast, however, efforts are being taken to implement it for higher eukaryotes. Also 'reverse ChIP' in which all proteins bound to a given DNA fragment could be identified in a single experiment is a major future challenge. Finally methods relying on big amount of sequence data such as 'phylogenetic footprinting' will most likely flourish due to development of

'next-generation' sequencing technologies (reviewed by Busch and Lohmann, 2007). Hopefully this new technologies will also benefit in better understanding of the DOF TF family.

7. References

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8 Supplementary materials

8.1 Supplementary materials for chapter 3: "DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in Arabidopsis"

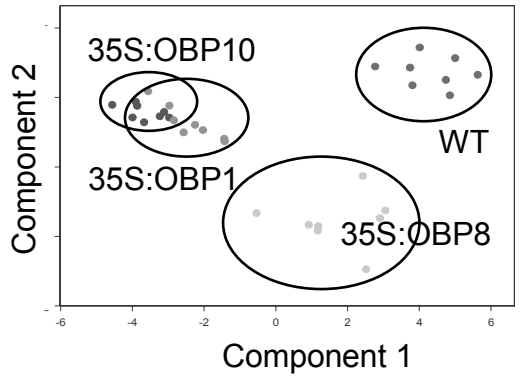

Figure S1. Principal component analysis of metabolite profiles of wild-type and *35S:OBP2* plants. The distances between these populations were calculated as described in Roessner *et al.* (2001). Each data point represents an independent sample.

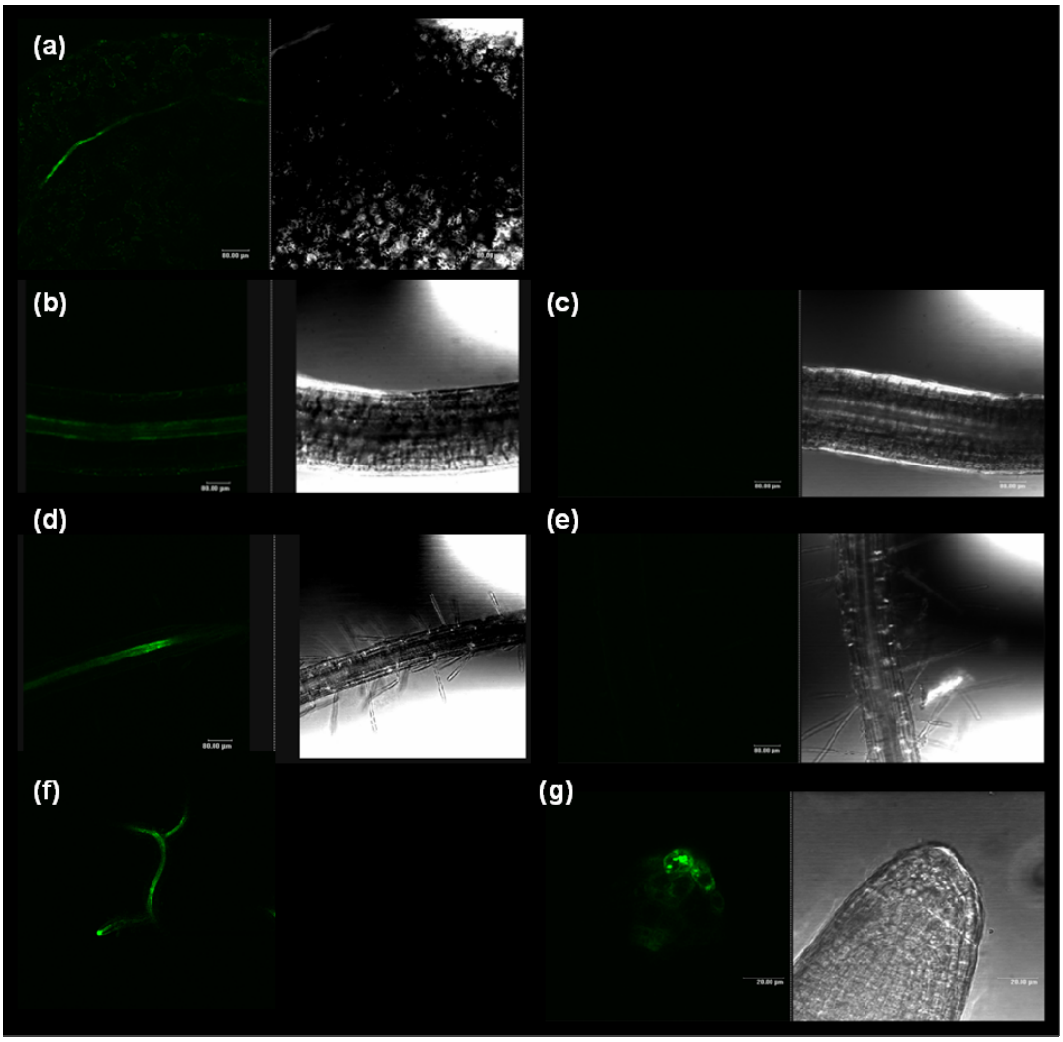

Figure S2. Analysis of *35S:OBP2* / *DR5α:GFP* plants. *35S:OBP2* (a,b,d,f) and wild-type plants (C24) (c,e,g) were transformed with auxin reporter construct DR5α-GFP kindly provided by Prof. Gert Jürgens (University of Tübingen). Seedlings were analyzed using confocal microscopy. (a) Cotyledon, notice GFP signal in the vasculature. (b, c) Hypocotyls. (d, e) Primary root. (f) Primary root with lateral roots. (g) Root apical meristem. (a-f) bar=80μm (g) bar=20 μm

Table S1

Genes differentially expressed between wild-type and *35S:OBP2* lines. Genes were considered to exhibit altered expression level when the hybridization signal was >2-fold increased or decreased in all comparisons to wild-type or transgenic (empty-vector) control lines.

Affymetrix code	AGI code	Gene identification and putative function	Fold induction 35S#8	Fold induction 35S#10
16461_i_at	At2g38380	peroxidase	7.4	4.7
20227_s_at	At1g52030	myrosinase binding protein, putative	7.2	7.3
15189_s_at	At2g42840	protodermal factor 1	6.4	5.0
18823_s_at	At4g33260	fizzy-related (FZR), putative	6.1	3.6
13048_s_at	At2g02850	putative basic blue protein (plantacyanin)	5.6	3.9
19683_at	At1g62500	putative proline rich cell wall protein	5.4	5.6
15981_at	At3g16420	myrosinase binding protein, putative	5.2	3.7
12574_at	At3g60140	glycosyl hydrolase family 1-b-glucosidase	5.0	4.8
18231_at	At4g15910	drought induced protein D21	4.8	3.8
12748_f_at	At4g11320	cysteine proteinase	4.6	4.9
12356_at	At5g06720	peroxidase, putative	4.4	3.9
13495_s_at	At2g02850	putative basic blue protein (plantacyanin)	4.4	3.6
18789_at	At5g60890	Myb TF or receptor-like protein kinase (ATR1), putative	4.3	3.1
17273_at	At2g39330	putative myrosinase-binding protein	4.3	4.5
16557_s_at	At2g29890	putative villin	4.2	3.2
12332_s_at	At3g12500	glycosyl hydrolase family 19 (basic endochitinase)	4.2	5.0
13530_g_at	At2g25060	similar to early nodulins	4.2	3.2
20201_at	At4g20030	glycine-rich protein	4.1	2.9
16846_s_at	At3g22880	meiotic recombination protein (AtDMC1)	4.1	4.2
19762_at	At4g21680	peptide transporter - like protein	4.0	5.6
12240_at	At4g27860	expressed protein	3.7	3.2
12736_f_at	At1g06180	myb family transcription factor	3.7	2.7
13278_f_at	At5g12030	heat shock protein 17.6A	3.6	3.4
15125_f_at	At5g24780	vegetative storage protein Vsp1	3.6	5.7
12744_at	At3g16470	putative lectin	3.5	4.0
20328_at	At2g22420	putative peroxidase	3.5	3.2
12678_i_at	At1g14900	linker histone protein, putative	3.5	1.7
15141_s_at	At5g24770	vegetative storage protein Vsp2	3.5	5.2
19186_s_at	At3g50970	dehydrin Xero2	3.5	4.2
18607_at	At2g33850	expressed protein	3.4	2.1
16820_at	At3g48350	Expressed protein	3.4	2.7
13284_at	At1g56410	heat shock protein, putative	3.3	4.7
20252_at	At2g23010	putative serine carboxypeptidase I	3.3	3.2
14856_at	At2g34490	CYP710A2	3.2	3.0
15052_at	At2g33380	RD20 protein	3.2	3.1
13949_at	At4g17470	putative protein	3.0	4.3
14980_at	At1g14040	unknown protein	3.0	2.6
19187_at	At3g12110	actin 11	3.0	2.0
19839_at	At2g28790	putative thaumatin	2.9	2.1
13134_s_at	At2g47180	putative galactinol synthase	2.9	3.8
14117_at	At4g37410	CYP81F4	2.9	2.3
17070_at	At1g18250	pathogenesis-related group 5 protein, putative	2.9	1.8
20329_g_at	At2g22420	putative peroxidase	2.9	2.0
19560_at	At3g57600	AP2 transcription factor - like protein	2.9	3.3
19063_at	At4g03210	xyloglucan endotransglycosylase	2.9	2.8
15621_f_at	At2g22240	putative myo-inositol 1-phosphate synthase	2.7	2.5
13277_i_at	At5g12030	heat shock protein 17.6A	2.6	2.8
15328_at	At4g37590	putative protein	2.6	1.4
20641_at	At1g52690	late embryogenesis-abundant protein, putative	2.6	4.0
15971_s_at	At3g49120	putative lectin	2.5	3.0
14527_at	At2g30500	unknown protein	2.4	1.5
16173_s_at	At4g37430	CYP81F1	2.4	3.8
20096_at	At2g34810	FAD-linked oxidoreductase family	2.4	3.1
16524_at	At1g54100	aldehyde dehydrogenase homolog, putative	2.4	2.3
18696_s_at	At1g62180	5'-adenylylphosphosulfate reductase, putative	2.3	2.2
20220_at	At4g02280	sucrose synthase, putative	2.2	1.8
19549_s_at	At2g22330	CYP79B3	2.2	2.0
17632_at	At4g01440	hypothetical protein	2.2	2.7
17428_at	At4g37870	phosphoenolpyruvate carboxykinase (ATP) -like protein	2.2	1.7
19993_at	At1g78490	CYP708A3	2.2	1.6
12966_s_at	At4g34590	bZIP family transcription factor	2.1	2.7
12333_at	At4g36430	peroxidase, putative	2.1	1.9
18503_at	At4g17880	bHLH protein	2.0	1.5

12402_at	At2g34850	putative UDP-galactose-4-epimerase	2.0	2.1
13645_at	At1g05340	expressed protein	1.9	3.9
13603_f_at	At4g21650	subtilisin proteinase - like	1.9	1.9
14068_s_at	At2g36950	expressed protein	1.9	1.7
14062_at	At2g47780	unknown protein	1.8	1.5
18109_s_at	At2g23150	putative metal ion transporter (NRAMP)	1.8	1.3
20027_at	At1g50420	scarecrow-like 3	1.8	2.1
14542_i_at	At2g24280	putative prolylcarboxypeptidase	1.8	1.4
19106_at	At4g32980	homeobox gene ATH1	1.8	1.4
16639_s_at	At1g69700	AtHVA22c	1.7	1.5
17485_s_at	At4g16260	glycosyl hydrolase family 17	1.6	1.3
18222_at	At2g18160	bZIP family transcription factor	1.6	1.5
15523_s_at	At4g24350	unknown protein	1.6	1.6
13895_at	At2g39420	putative phospholipase	1.6	2.0
15672_s_at	At2g22470	arabinogalactan protein AGP2	1.6	4.2
17983_at	At5g42970	COP9 complex subunit, FUS4	1.6	1.3
12027_at	At4g20170	putative protein	1.6	2.2
18497_at	At2g32120	70kD heat shock protein	1.5	1.6
14673_s_at	At4g27070	tryptophan synthase beta-subunit (TSB2)	1.5	1.2
13192_at	At5g51460	trehalose-6-phosphate phosphatase	1.5	1.9
14001_at	At3g24800	E3 ubiquitin ligase, PRT1	1.5	1.2
20263_at	At5g21105	ascorbate oxidase, putative	1.5	2.1
14539_s_at	At2g42490	putative copper amine oxidase	1.3	1.9
17119_s_at	At2g06050	12-oxophytodienoate reductase (OPR3)(DDE1)	1.3	2.0
16611_at	At5g13550	sulfate transporter	1.3	1.6
19591_at	At1g44350	IAA-aminoacid hydrolase	1.3	1.6
17342_at	At1g77210	sugar carrier protein, putative	-1.1	-1.8
13143_at	At2g03440	unknown protein	-1.3	-1.2
19617_at	At1g34760	14-3-3 protein GF14 omicron (grf11)	-1.3	-2.5
13538_at	At4g20780	calcium-binding protein - like	-1.4	-1.1
16076_s_at	At2g17460	pseudogene, similar to hypothetical protein GB:AC005825	-1.4	-1.2
12577_at	At2g28630	putative fatty acid elongase	-1.4	-2.5
14732_at	At1g62380	ACC oxidase, putative	-1.5	-3.6
12998_at	At3g47800	aldose 1-epimerase - like protein	-1.5	-1.9
16986_at	At4g22540	putative protein	-1.7	-1.6
13985_at	At2g25930	unknown protein	-1.7	-2.0
17207_at	At4g36670	mannitol transporter	-1.8	-2.8
12781_at	At1g13930	expressed protein	-1.8	-1.6
14554_at	At1g70420	expressed protein	-1.9	-2.2
12360_at	At4g23210	serine/threonine kinase - like protein	-1.9	-2.1
14310_at	At2g39400	putative phospholipase	-1.9	-1.9
17943_at	At2g28720	putative histone H2B	-1.9	-1.7
20150_at	At5g27280	putative protein	-1.9	-1.4
13172_s_at	At1g20840	putative sugar transporter protein	-1.9	-1.5
14472_at	At5g05320	monooxygenase 2 (MO2), putative	-2.0	-1.9
17003_at	At2g39900	putative LIM-domain protein	-2.0	-1.9
16053_i_at	At5g02200	hypothetical protein	-2.0	-1.9
18556_at	At2g21130	cyclophilin CYP2	-2.1	-1.9
12642_at	At2g15390	xyloglucan fucosyltransferase, putative	-2.1	-1.6
12236_at	At3g47860	putative protein	-2.1	-1.9
17689_at	At1g60360	hypothetical protein	-2.2	-1.7
14123_at	At2g45660	MADS-box protein (AGL20)	-2.2	-2.1
18665_r_at	At2g20260	putative photosystem I reaction center subunit IV	-2.3	-2.8
16502_at	At1g20010	beta tubulin 1, putative	-2.4	-2.4
14608_at	At1g13210	potential phospholipid-transporting ATPase 11	-2.5	-1.7
15663_s_at	At1g13260	DNA-binding protein (RAV1)	-2.9	-1.5
12768_at	At2g15890	expressed protein	-3.1	-3.1
13785_at	At2g42530	cold-regulated protein cor15b precursor	-3.2	-3.1
14649_at	At1g12220	disease resistance protein RPS5 (resistance to Pseudomonas	-3.2	-3.1
12861_s_at	At5g06690	thioredoxin-like	-3.3	-2.5
15558_r_at	At4g20410	putative protein	-3.4	-4.0
19359_at	At4g32340	putative protein	-3.6	-3.2
19644_at	At5g66850	Map3kgamma protein kinase	-3.7	-1.9
15105_s_at	At2g21660	glycine-rich RNA binding protein AtGRP7	-3.7	-2.8
18272_at	At2g40080	expressed protein	-4.1	-3.0
18296_at	At2g41560	potential calcium-transporting ATPase 4, plasma membrane-	-4.2	-2.4
12364_at	At3g57240	glycosyl hydrolase family 17 (beta-1,3-glucanase bg3)	-4.5	-4.5
18258_at	At2g18210	unknown protein	-4.6	-3.8
15422_at	At4g04330	expressed protein	-4.6	-3.6

Table S2. Gene expression data for selected genes connected to plant stress responses.

AGI code	Gene function	Fold change[a] (log_2)	
		35S:OBP2#8	*35S:OBP2#10*
At1g52030	myrosinase-binding protein, putative	7,2	7,3
At3g16420	myrosinase-binding protein, putative	5,2	3,7
At2g39330	myrosinase-binding protein, putative	4,3	4,5
At5g60890	ATR1, myb family transcription factor	4,3	3,1
At4g27070	tryptophan synthase beta-subunit (TSB2)	1,5	1,2
At2g22330	cytochrome P450 enzyme, CYP79B3	2,2	2,0
At4g39950	cytochrome P450 enzyme, CYP79B2	3,3	2,8
At2g02850	putative basic blue protein (plantacyanin)	5.0	3,7
At3g12500	glycosyl hydrolase family 19 (basic endochitinase)	4,2	5,0
At1g06180	myb family transcription factor	3,7	2,7
At5g24780	vegetative storage protein Vsp1	3,6	5,7
At3g16470	putative lectin	3,5	4,0
At5g24770	vegetative storage protein Vsp2	3,5	5,2
At2g28790	putative thaumatin	2,9	2,1
At1g18250	pathogenesis-related group 5 protein, putative	2,9	1,8
At3g49120	putative lectin	2,5	3,0
At2g34810	FAD-linked oxidoreductase family	2,4	3,1
At1g69700	AtHVA22c	1,7	1,5
At4g16260	glycosyl hydrolase family 17	1,6	1,3
At2g06050	12-oxo-phytodienoic acid reductase (OPR3) (DDE1)	1,3	2,0
At1g13260	DNA-binding protein (RAV1)	-2,9	-1,5
At1g12220	disease resistance protein RPS5	-3,2	-3,1
At3g57240	glycosyl hydrolase family 17 (beta-1,3-glucanase bg3)	-4,5	-4,5
At4g15910	drought induced protein D21	4,8	3,8
At2g25060	similar to early nodulins	4,2	3,2
At5g12030	heat shock protein 17.6A	3,6	3,4
At3g50970	dehydrin Xero2	3,5	4,2
At1g56410	heat shock protein, putative	3,3	4,7
At2g33380	RD20 protein	3,2	3,1
At2g47180	putative galactinol synthase	2,9	3,8
At2g22240	putative myo-inositol 1-phosphate synthase	2,7	2,5
At3g57600	AP2 transcription factor - like protein	2,9	3,3
At5g12030	heat shock protein 17.6A	2,6	2,8
At4g02280	sucrose synthase, putative	2,2	1,8
At2g23150	putative metal ion transporter (NRAMP)	1,8	1,3
At2g32120	70kD heat shock protein	1,5	1,6
At1g62380	ACC oxidase, putative	-1,5	-3,6
At2g42530	cold-regulated protein cor15b precursor	-3,2	-3,1

[a] Fold change is an average value of two pair-wise comparisons to wild-type and control plants, respectively, and is given as log_2 value.

Table S3. Metabolite levels in wild-type and *35S:OBP2* leaves of Arabidopsis. Values are the mean ± SE of eight independent determinants and are presented as x-fold changes compared to wild-type. To assess the significance a Student´s t-test was performed and included in the table.

	WT	35S:OBP2#1			35S:OBP2#10			35S:OBP2#8		
	average response	average response	x-fold	t-test	average response	x-fold	t-test	average response	x-fold	t-test
Succinic acid	152.43	83.03	0.54	0.001	84.91	0.56	0.001	112.15	0.74	0.029
Raffinose	24.09	172.89	7.18	0.000	77.27	3.21	0.000	17.61	0.73	0.293
Galactinol	29.20	313.66	10.74	0.000	134.04	4.59	0.000	15.92	0.55	0.149
Trehalose	66.03	13.49	0.20	0.189	16.04	0.24	0.210	18.20	0.28	0.230
Sucrose	41.50	117.54	2.83	0.000	102.80	2.48	0.000	53.67	1.29	0.107
Glucose-6-phosphate	0.97	1.81	1.87	0.000	1.73	1.78	0.002	1.14	1.18	0.436
trans-Sinapinic acid	15.54	29.26	1.88	0.000	28.53	1.84	0.000	20.81	1.34	0.047

Uric acid	2.03	6.97	3.44	0.024	7.27	3.59	0.013	5.76	2.84	0.006
myo-Inositol	216.70	940.10	4.34	0.000	765.25	3.53	0.000	504.64	2.33	0.000
cis-Sinapinic acid	5.46	8.06	1.48	0.000	7.15	1.31	0.000	5.84	1.07	0.292
Galactonic acid	10.55	10.34	0.98	0.612	9.62	0.91	0.075	9.99	0.95	0.272
L(+)-Ascorbic acid	282.62	399.33	1.41	0.000	396.40	1.40	0.000	353.72	1.25	0.009
L-Tyrosine	2.95	10.54	3.58	0.000	8.40	2.85	0.001	3.89	1.32	0.451
L-Lysine	2.87	10.32	3.60	0.000	8.09	2.82	0.000	6.22	2.17	0.007
Glucose methoxyamine	80.37	83.93	1.04	0.766	79.84	0.99	0.964	45.60	0.57	0.009
D-Gluconic acid-1,5-lactone	4.16	13.21	3.18	0.002	11.30	2.72	0.002	5.24	1.26	0.158
Fructose methoxyamine	28.21	20.14	0.71	0.077	17.11	0.61	0.013	10.41	0.37	0.001
Dehydroascorbic acid dimer	95.62	200.47	2.10	0.000	175.71	1.84	0.000	153.44	1.60	0.003
Citric acid	5648.8	5493.6	0.97	0.660	5291.0	0.94	0.315	6118.3	1.08	0.299
Shikimic acid	33.57	36.74	1.09	0.067	37.22	1.11	0.096	27.16	0.81	0.001
L-Glutamine	3.69	24.19	6.55	0.001	27.38	7.42	0.001	15.27	4.14	0.252
L-Glycerol-3-phosphate	1.90	4.71	2.48	0.000	3.86	2.03	0.003	2.25	1.18	0.320
cis-Aconitic acid	2.17	1.67	0.77	0.001	2.02	0.93	0.637	2.12	0.98	0.829
Putrescine	23.29	16.31	0.70	0.055	13.67	0.59	0.011	12.67	0.54	0.013
Ribose methoxyamine	3.21	5.20	1.62	0.004	4.99	1.55	0.003	2.96	0.92	0.637
L-Asparagine	2.82	2.96	1.05	0.667	3.00	1.06	0.541	3.76	1.33	0.006
Arabinose methoxyamine	1.19	2.39	2.01	0.000	1.64	1.38	0.172	1.28	1.08	0.526
Xylose methoxyamine	5.95	12.94	2.18	0.000	11.37	1.91	0.000	8.36	1.41	0.007
L-Glutamic acid	213.69	268.54	1.26	0.232	286.92	1.34	0.154	301.88	1.41	0.086
L-Phenylalanine	5.33	14.95	2.81	0.000	11.72	2.20	0.000	8.32	1.56	0.005
2-Ketoglutaric acid	3.95	2.67	0.67	0.000	2.33	0.59	0.000	2.12	0.54	0.000
Threonic acid	13.03	12.03	0.92	0.154	11.99	0.92	0.118	13.37	1.03	0.682
L-Cysteine	1.25	1.55	1.24	0.006	1.46	1.17	0.020	1.36	1.09	0.395
Pyroglutamic acid	607.13	670.65	1.10	0.263	715.11	1.18	0.128	679.79	1.12	0.160
4-Aminobutyric acid	13.73	19.21	1.40	0.274	14.92	1.09	0.803	12.03	0.88	0.722
L-Aspartic acid	468.99	542.09	1.16	0.279	539.43	1.15	0.033	660.94	1.41	0.000
L-Methionine	2.71	4.51	1.67	0.011	4.29	1.59	0.000	3.44	1.27	0.022
Erythritol	4.35	7.56	1.74	0.000	7.82	1.80	0.009	8.11	1.86	0.117
Salicylic acid	1.19	1.03	0.87	0.377	1.06	0.89	0.434	0.93	0.78	0.205
Malic acid	1077.1	1501.7	1.39	0.000	1599.2	1.48	0.000	1122.2	1.04	0.593
beta-Alanine	3.84	7.60	1.98	0.000	6.63	1.73	0.000	6.15	1.60	0.002
Threonic acid-1,4-lactone ,	3.87	4.53	1.17	0.025	4.48	1.16	0.034	3.96	1.02	0.788
Fumaric acid	641.34	307.36	0.48	0.000	425.06	0.66	0.015	1407.3	2.19	0.000
L-Alanine	58.27	44.50	0.76	0.276	46.23	0.79	0.344	41.76	0.72	0.165
DL-Alanine-2,3,3,3-d4	335.90	212.11	0.63	0.098	228.58	0.68	0.169	293.11	0.87	0.588
Uracil	1.20	2.17	1.81	0.000	2.01	1.67	0.001	2.30	1.91	0.003
Glyceric acid	5.93	9.19	1.55	0.003	8.76	1.48	0.009	5.85	0.99	0.936
Maleic acid	4.80	4.98	1.04	0.788	4.88	1.02	0.902	4.75	0.99	0.928
Glycine	600.88	60.80	0.10	0.000	120.92	0.20	0.000	16.15	0.03	0.000
Nicotinic acid	1.10	2.90	2.63	0.000	2.52	2.29	0.000	2.11	1.92	0.001
L-Proline	28.72	667.77	23.25	0.000	655.64	22.83	0.000	239.43	8.34	0.010
L-Threonine	60.59	174.67	2.88	0.000	166.24	2.74	0.000	104.46	1.72	0.000
L-Isoleucine	8.81	30.57	3.47	0.000	25.07	2.85	0.000	19.99	2.27	0.000
Glycerol	38.74	37.76	0.97	0.807	48.00	1.24	0.158	34.47	0.89	0.227
Phosphoric acid	311.85	267.38	0.86	0.386	274.13	0.88	0.451	249.61	0.80	0.223
L-Leucine	8.65	28.82	3.33	0.000	22.54	2.61	0.000	18.72	2.16	0.001
L-Serine	161.35	664.83	4.12	0.000	565.50	3.50	0.000	199.76	1.24	0.173
Benzoic acid	4.60	3.38	0.74	0.020	3.57	0.78	0.073	3.31	0.72	0.021
L-Valine	33.30	91.85	2.76	0.000	76.40	2.29	0.000	56.41	1.69	0.000
Lactic acid	50.97	35.35	0.69	0.021	49.25	0.97	0.855	37.41	0.73	0.076

Table S4. Glucosinolate levels in transgenic plants.

Glucosinolates were determined using HPLC. Data are means ± SD (n=3). Samples were replicated with individual plants from one experiment. Asterisks (*) indicate values that are significantly different ($p < 0.05$) in comparison to respective controls. K#1 and K#2 are independent 35S empty-vector control lines. Concentrations are given in µmol/g fresh weight (values shown are means ± SD; n = 3). 3MSOP, 3-Methylsulfinylpropyl; R-2OH-3B, (2*R*)-2-Hydroxy-3-butenyl; S-2OH-3B, (2*S*)-2-Hydroxy-3-butenyl; Allyl, 2-Propenyl; 3-Butenyl, 3-butenyl; 7MSOH, 7-Methylsulfinylheptyl; 4-Pentenyl, 4-Pentenyl; 8MSOO, 8-Methylsulfinyloctyl; I3M, Indol-3-ylmethyl; 4MOI3M, 4-Methoxyindol-3-ylmethyl; ; 7MTH, 7-Methylthioheptyl; 8MTO, 8-Methylthiooctyl.

	C24	K#1	K#2	*35S:OBP2#1*	*35S:OBP2#8*	*35S:OBP2#10*
From short chain methionine derivatives						
3MSOP	0.15±0.005	0.15±0.05	0.15±0.05	0.19±0.012	0.16±0.04	0.09±0.009
R-2OH-3B	0.01±0.0015	0.018±0.011	0.01±0.003	0.016±0.007	0.013±0.003	0.014±0.002
S-2OH-3B	0.012±0.001	0.02±0.002	0.015±0.005	0.12±0.04*	0.041±0.009*	0.18±0.02*
Allyl	0.18±0.013	0.17±0.038	0.17±0.055	0.39±0.08*	0.4±0.1*	0.42±0.02*
3-Butenyl	1.25±0.15	1.54±0.12	1.32±0.57	2.6±0.29*	2.2±0.51	2.45±0.14*
4-Pentenyl	0.017±0.001	0.019±0.002	0.019±0.01	0.03±0.002	0.032±0.01	0.043±0.003*
From long-chain methionine derivatives						
7MTH	0.011±0.006	0.016±0.008	0.019±0.012	0.023±0.02	0.005±0.005	0.013±0.012
8MTO	0.028±0.012	0.038±0.025	0.027±0.01	0.046±0.024	0.024±0.002	0.037±0.014
7MSOH	0.19±0.009	0.18±0.04	0.19±0.033	0.19±0.007	0.19±0.03	0.18±0.01
8MSOO	0.64±0.019	0.69±0.11	0.64±0.15	0.78±0.1	0.7±0.12	0.7±0.05
From tryptophan						
I3M	0.063±0.007	0.063±0.009	0.058±0.024	0.163±0.021*	0.19±0.034*	0.2±0.045*
4MOI3M	0.024±0.004	0.03±0.012	0.024±0.006	0.009±0.002*	0.008±0.002*	0.007±0.002*

8.2 Supplementary materials for chapter 4. "Transcription factor AtDOF4;2 affects phenylpropanoid metabolism in *Arabidopsis thaliana*"

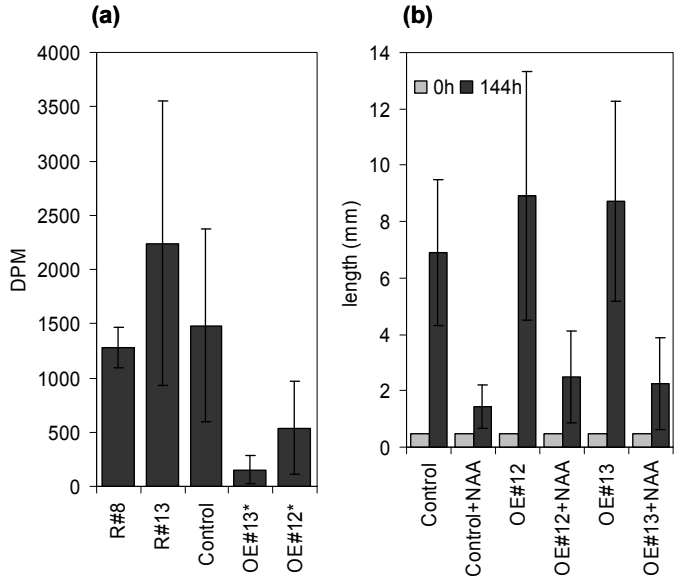

Figure S1. Analysis of auxin transport and auxin signaling in *35S-AtDOF4;2* plants. (a) Basipetal auxin transport was measured as described in Brown *et al.* (2001) using 5h incubation time. Data are means ± SD (n=6-9); stem segments were from one experiment replicated with individual plants. (b) Sensitivity of nodes to apical application of auxin (2.5μM NAA) was measured as described in Chatfield *et al.* (2000). Data are means ± SD (n=6-11); bud containing stem segments were from independent plants from one experiment. Graph represents length of the secondary stem grown from the bud. *Asterisks* (*) indicate values that are significantly different from the control plants (Student´s t-test; $p<0.05$).

Table S1. List of primers used in Q-RTPCR experiments.

AGI code	Gene Name	Forward Primer	Reverse Primer
At4g21030	*DOF4;2*	TGAAATGAATGTGATGCCCCCACC	GCGCGGTTGAGACTTCTTGTTGTT
At3g18780	*Actin2*	TCCCTCAGCACATTCCAGCAGAT	AACGATTCCTGGACCTGCCTCATC
At4g05320	*UBQ10*	CACACTCCACTTGGTCTTGCGT	TGGTCTTTCCGGTGAGAGTCTTCA
At1g65060	*4-Coumaroyl-CoA ligase 3*	TTCGTGGTGCGATCAAATGG	GAACACCACCTGTTTGGCAA
At5g13930	*Chalcone synthase*	AAGCGCATGTGCGACAAGTC	AGAAGGAGCCATGTAAGCAC
At3g55120	*Chalcone isomerase*	CTCTCTTACGGTTGCGTTTTC	GTTCTTCCCGATGATAGATTC
At3g51240	*Flavonol-3-hydroxylase*	ACCACGGCCATTTTTTGAGC	CGTGGCTATGGATAATCTGC
At5g07990	*Flavonol-3¢-hydroxylase*	CTTACCTTCAGGCGGTTATC	ATCTCACAGCTCTCTGACGC
At5g08640	*Flavonol synthase*	CGATCAGATTCTGAGGTTGAG	CACGACATCCTCGTCTTCTC
At5g42800	*Dihydroflavonol reductase*	AAAGATCCTGAGAACGAAGTG	ATCTTCGTACGGTCTTTGCC
At5g17220	*Glutathione-S-transferase*	GAACATCTTCTTCGTCAGCC	GTAGTATCTCGCGATGGCTC
At4g22880	*Leucoanthocyanidin dioxygenase*	AAGCTCACACCGATGTAAGC	TTTGCAGTGACCCATTTGCC
At1g56650	*PAP1*	TGTAAGAGCTGGGCTAAACC	GAAGATCGACTTCATCAGAGC
At2g37040	*Phenylalanine ammonia lyase*	GAAGCTTCACGAGATGGATC	TGACGGAGTTAATCTCACGC
At3g53260	*Phenylalanine ammonia lyase*	TACTCGACGGAAGCTCATAC	GTACGAAGAGCGTAACGATC
At5g04230	*Phenylalanine ammonia lyase*	CAAGATCGTTACGCTCTGCG	TTCGATCATCTTAGTCGCTGC
At3g10340	*Phenylalanine ammonia lyase*	CCTAAACAAGATCGGTACGC	ACAGAGTTGATCTCACGCTC
At2g30490	*Cinnamate-4-hydroxylase*	CCGCGATTGAGACAACATTG	AACTGTGTCGAGTTCGTTCC
At2g40890	*Coumarate-3-hydroxylase*	TCTATGGGATATGATCACGGC	GGATTCTTGATCATTTCCGCC
At3g21230	*4-Coumaroyl-CoA ligase 5*	CCGCAGTTGTTGCAATGAAG	CTGAGACCCTTGAGATCT TG
At1g51680	*4-Coumaroyl-CoA ligase 1*	AGCTGGTGAAGTTCCTGTTG	ACACAACCTGTTTCGACACG
At3g21240	*4-Coumaroyl-CoA ligase 2*	GTCGTCGCCATGAAGGAAG	GATCTCACCACAAACGCAAC
At4g34050	*Caffeoyl-CoA O-methyltransferase 1*	CTACGACAACACTCTGTGGA	GGAGCATACAGATCTCGATC
AT4G26220	*Caffeoyl-CoA O-methyltransferase 2*	GCAAGGTTATCGCCATTGAC	ACCGGCAATCCTATTTCATAC
AT1G67980	*Caffeoyl-CoA O-methyltransferase 4*	TTGGTGAACGACAAATGTGAG	CTTGATTTGTCAGCATCCGC
At5g48930	*Hydroxycinnamoyltransferase*	CTTCTCGTTTTGCAGGTGAC	TGTTGCATCCCAACCCCAAG
At4g36220	*Ferulate-5-hydroxylase*	TCCGACATCGAGAAGTTGAC	AACCGTCGATACTAGTGTCC

8.3 Supplementary materials for chapter 5: "DOF transcription factor OBP1 (AtDOF3.4) is involved in cell cycle regulation"

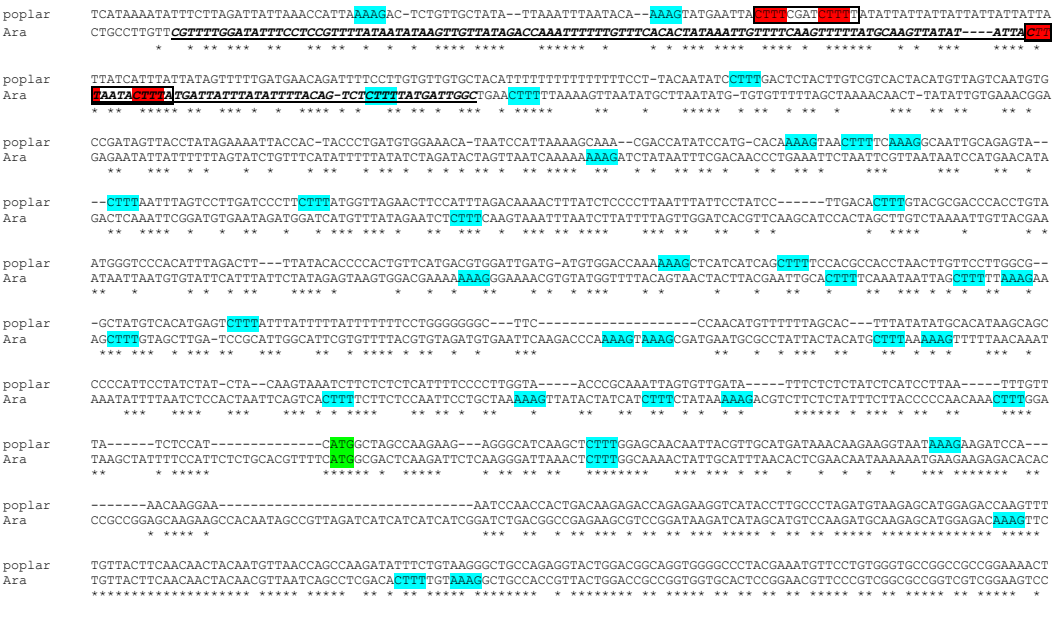

Figure S1. Promoter analysis of *AtDOF2;3* ortholog from poplar. Poplar ortholog of *AtDOF2;3* was retrieved from Plant Tanscription Factor Databases website (http://planttfdb.cbi.pku.edu.cn/). Sequence comparison was performed by ClustalW (http://www.ebi.ac.uk/Tools/clustalw/). Blue shading indicates

DOF binding sites, green shading the start codon ATG and red consensus tandem DOF elements found in promoter fragments enriched in ChIP experiments (underlined).

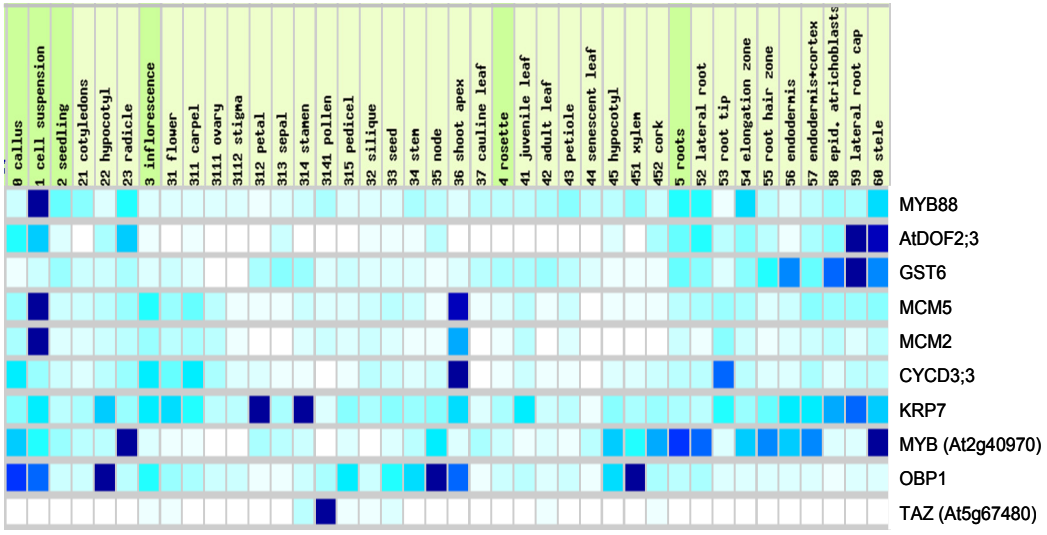

Figure S2. Meta-analysis of organ specific expression of *OBP1*, its early targets and *GST6* was performed using the Meta-Analysis tool available at the Genevestigator website (Zimmermann *et al.*, 2004; Schmid *et al.*, 2005).

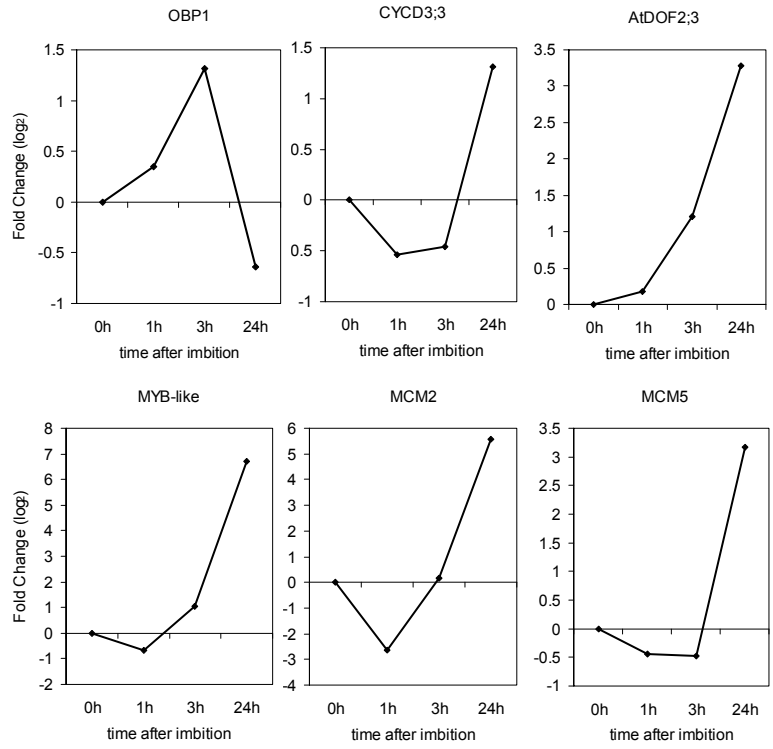

Figure S3. Expression of *OBP1* and its early targets is associated with germination. Graphs represent fold change (log_2) of expression of *OBP1* and its early targets during seed imbibition. Data were

retrieved from two germination experiments of the AtGenExpress project (Expression profiling of early germinating seeds and Effect of ABA during seed imbibition, Kamiya and Nambara).

```
Ath  AACTGAAAACTTTGAAAAC-AGAGCCATCTACGTATGAAGAGAGCCGACT-----TCATAGGGATGAAAAACAGAGCATTTCACTGTTTACAATAATCCCATAGCCGACTCAACAATTCC
Br   AACAGAAAGTCTTGAAAGA-AGAGCCACCTACGTATGAAGTGAGCCGAGTCGTAGTTGTAGGGA--GCAAACAGAGC-CAACACTGCTCACAATAATCC-ATAGCCGACTCAACAATTCC
Pop  AGCCGCATACCTAAGCTGCTAGAGCTGACTAGCTGACCCCAGTAAGAAACC-CGTTTCTGGAG----CCGACTGAATATGTCGTGCCTGGC--TACCCCTATAACAGA----ACACTGTA
     * * * *    *        *****   ***  *       *     *       *  * * *       ** **      *     *  *  **  ** *** * **    *** *

Ath  AAAGCTAAACAATAATAAACAAATCCTCATAAAAAGACAAATTTCAAAGACAAGAGAGAAGAGAGAGATAGAGAGCGTGGCCTCTAATCAACCCAACACCCTCTGTCAAAAACAAGCCTG
Br   AAA----------------------------AAAAAACAAACTTTA----------------GAGAGAGAGAGAGCGT-----CTAATCAACCCAACACCCTCTGTCAAGAA-AAGTCTG
Pop  AAAGCTGCTGAA---------GCTGCTGCTGCTGCTGCTGCTCTCTCTCTCTCTCTCTT---CCCTGCCTTGCACCGCACCCTCTCCTCATACCCTCAGAATTTTTCAG-----ATTCTC
     ***                                  *     *                       *      * **      **  ***  **  **   * * ***      *  **

Ath  ACTTTCTTCTCCATCCCCATTTCTATCTGCGCATGTCTCTCTCTTTCTTCTC-CATAAAAAAC---CAAATTCT-CACTCTCTCATACCCTAAAGTTTCAACCTTTAAACCCTAAAACCA
Br   ACTTTCTTCTCCATCACCATCTCTATCTGCGCATGTTTCCTCCATAAAAACC-AAAACAAAACTCCCCAAATCT-CACTCCCTCGTACCA-AAAGTTCCAACCTTTTACCC---------
Pop  CCTCTCCCATCTATCTCTACATAAATTCCCCTCTTTTACCCCCTTGCCACCCACATTTCACATCCTCACAAGCTACACGCGCATTCACAGAAAATCACTCTTCTTTCTATCTTCAAACAC
      ** **   ** *** * *  *  **   *   * *  *   * *      *  *    * *    *  *  ** *** * *    **   ***        ****    *

Ath   AAAAAGCTTCAGTCTTTTGCATCACTTCCCTTAAAGCTTCAACCTTTCTC----CGATTATGCCGACG------TCTGACTCCGGTGAACCACGGCGA--ATAGCTAT-GAAACCTAACG
Br    -AAAAGCCCCAATCTTTC---TTCTTTCCC-----GCGTC-----TTCT--------TGATGACGACG------TCTGACTCCGGCGAATCACGACGC--ACGGCGAT-GAGACCTCACG
Pop   GACAAAACATAACCAATA--CTCACTCCTCCAACACTCTCTTTGCCCCCAAAAGCCAAAATGCCGGCAGAATTATCAAGCAGCGAAGCAGCAAGGAGAGCACAACAATCGACAGCTGCAA
       * **     *  *  *    *   * * *        **       *           *** ** *       **   *  **  * * ** *  *   *   * ** ** * **

Ath   GCGTAACAGT---------TCCGATTTCTGACCAGCAAGAACAGCTTCCATGTCCTCGTTGTGACTCATCCAACACTAAGTTCTGTTACTACAACAACTACAACTTCTCTCAGCCTCGTC
Br    GCGGAATAACGGGGCTTCCACCGGCCACGGAGCAGCAAGAGCAGCTCCCGTGTCCTCGCTGCGACTCAGCCAACACCAAGTTCTGTTACTACAACAACTACAACTTCTCCCAGCCTCGCC
Pop   CTTTAACTAAGCCAGGAGGAGCACCACCTCAAGAACAAGAGCACTTGCCATGTCCACGTTGTGACTCAACGAATACTAAGTTTTGTTACTACAACAACTACAACTTCTCTCAGCCTCGTC
          **               *     *  *  * ***** **  * ** ***** ** ** ****** * ** ** ***** ************************** ******** *

Ath   ACTTCTGCAAAGCTTG-TCGTCGTTACTGGACTCACGGTGGTACTCTCCGTGACGTTCCT
Br    ACTTCTGCAAATCCTGGTCGCCGTTACTGGACTCACGGCGGAACTCTCCGCGA-------
Pop   ACTTCTGTAAGTCTTG-CCGCCGTTACTGGACTCACGGTGGCACCCTCCGGGACATTCCT
      ******* **  * **  ** ***************** ** ** ***** **
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Figure S4. Promoter analysis of *OBP1* and its orthologs from poplar and *Brassica oleracea.* In an attempt to identify conserved factors up-stream of *OBP1* we looked for orthologous genes in the closely related species *Brassica oleracea*. TIGR (The Institute for Genome Research) and the CSHL/WU (Cold Spring Harbor Laboratory/Washington University) consortium are currently performing genome shotgun sequencing with a goal of reaching 0.5 - 1x coverage of the *Brassica oleracea* genome. In parallel they are fully sequencing six Brassica BAC contigs identified by O'Neill and Bancroft in 2000 as being homeologous to a region of the Arabidopsis genome that is duplicated between chromosomes 4 and 5. A BLAST server at TIGR allows retrieval of sequences from the preliminary contigs and we used it to look for sequences similar to *OBP1* and its up-stream region. We found a 819 bp long fragment homologous to *OBP1* ORF, 5' UTR and approximately 400bp of promoter region. Information on the OBP1 ortholog from poplar was retrieved from Plant Tanscription Factor Databases website (http://planttfdb.cbi.pku.edu.cn/). Sequence comparison was performed by ClustalW (http://www.ebi.ac.uk/Tools/clustalw/). Red shading indicates the putative ESR2 binding site, green shading indicates the start codon ATG.

Table S1. Top 48 genes co-expressed with *OBP1*. Co-expression analysis was performed using the Arabidopsis Co-expression Tool (Manfield *et al.,* 2006).

AffyID	Locus	Description	Correlation
245828_at	AT1G57820	VIM1	0.85
252785_at	AT3G42660	nucleotide binding	0.84
255449_at	AT4G02820	pentatricopeptide (PPR) repeat-containing protein	0.84
254217_at	AT4G23720	unknown protein	0.84
249060_at	AT5G44560	SNF7 family protein	0.83
248861_at	AT5G46700	TRN2 (TORNADO 2)	0.83
256480_at	AT1G33410	hypothetical protein	0.81
256480_at	AT1G33410	hypothetical protein	0.81
260484_at	AT1G68360	zinc finger protein-related	0.81
249352_at	AT5G40430	MYB22	0.81
251147_at	AT3G63480	kinesin heavy chain	0.80
255481_at	AT4G02460	DNA mismatch repair protein	0.80
250212_at	AT5G13960	SUVH4	0.80
247028_at	AT5G67100	DNA-directed DNA polymerase alpha catalytic subunit	0.80
255419_at	AT4G03230	S-locus lectin protein kinase	0.80
255562_at	AT4G02070	MSH6	0.80
252349_at	AT3G48150	APC8	0.80
248891_at	AT5G46280	DNA replication licensing factor	0.80
267506_at	AT2G45520	hypothetical protein	0.79
264027_at	AT2G03670	ATPase	0.79
245147_at	AT2G45280	ATRAD51C	0.79
249997_at	AT5G18620	CHR17	0.79
261655_at	AT1G01940	peptidyl-prolyl cis-trans isomerase	0.79
247056_at	AT5G66750	DDM1	0.78
253771_at	AT4G28430	reticulon family protein	0.78
266959_at	AT2G07690	minichromosome maintenance family protein	0.78
246882_at	AT5G26180	NOL1/NOP2/sun family protein	0.78
247944_at	AT5G57100	transporter-related	0.78
251035_at	AT5G02220	hypothetical protein	0.78
266735_at	AT2G46930	pectinacetylesterase	0.78
247310_at	AT5G63950	CHR24	0.78
265422_at	AT2G20800	NDB4 (NAD(P)H DEHYDROGENASE B4)	0.78
251822_at	AT3G55060	similar to unknown protein	0.78
263926_at	AT2G21800	similar to unknown protein	0.77
250391_at	AT5G10850	similar to nucleic acid binding / zinc ion binding	0.77
261330_at	AT1G44900	DNA-dependent ATPase	0.77
255434_at	AT4G03180	similar to hypothetical protein	0.77
260528_at	AT2G47260	WRKY23	0.77
252540_at	AT3G45740	hydrolase family protein	0.77
266758_at	AT2G46920	POL (POLTERGEIST)	0.77
246536_at	AT5G15920	structural maintenance of chromosomes	0.77
246683_at	AT5G33300	chromosome-associated kinesin-related	0.77
258725_at	AT3G09620	DEAD/DEAH box helicase, putative	0.77
246910_at	AT5G25800	exonuclease family protein	0.76
245739_at	AT1G44110	CYCA1;1 (CYCLIN A1;1)	0.76
255520_at	AT4G02230	60S ribosomal protein L19 (RPL19C)	0.76
255507_at	AT4G02150	ATIMPALPHA3/MOS6; protein transporter	0.76
248279_at	AT5G52910	ATIM (TIMELESS)	0.76

Table S2. Up-regulated transcripts. Genes differentially expressed between DEX treated empty vector control and *IOE-OBP1* plants. Genes were considered to exhibit an altered expression level when the hybridization signal was >2-fold increased in the comparison between the two sets of plants.

AffyID	Locus	Change (log_2)	AffyID	Locus	Change (log_2)	AffyID	Locus	Change (log_2)
252210_at	AT3G50410	5.42	248891_at	AT5G46280	1.64	259922_at	AT1G72770	1.23
252691_at	AT3G44050	4.01	257295_at	AT3G17420	1.64	262637_at	AT1G06640	1.22
246415_at	AT5G17160	3.83	259537_at	AT1G12370	1.64	261774_at	AT1G76260	1.22
249375_at	AT5G40730	3.50	247441_at	AT5G62710	1.64	259278_at	AT3G01160	1.22
249809_at	AT5G23910	3.43	255991_at	AT1G29820	1.63	259863_at	AT1G72630	1.22
263258_at	AT1G10540	3.42	260510_at	AT1G51580	1.63	258609_at	AT3G02910	1.21
247038_at	AT5G67160	3.40	246475_at	AT5G16720	1.63	250212_at	AT5G13960	1.21
248691_at	AT5G48310	3.40	251281_at	AT3G61640	1.62	254821_at	AT4G12540	1.21
253978_at	AT4G26660	3.38	265339_at	AT2G18230	1.60	246399_at	AT1G58110	1.20
246557_at	AT5G15510	3.35	248055_at	AT5G55830	1.60	255557_at	AT4G01990	1.20
250823_at	AT5G05180	3.26	249332_at	AT5G40980	1.59	258871_at	AT3G03060	1.20
253804_at	AT4G28230	3.25	246345_at	AT3G56760	1.59	258119_at	AT3G14720	1.20
259978_at	AT1G76540	3.21	247056_at	AT5G66750	1.59	246724_at	AT5G29000	1.20
247039_at	AT5G67270	3.21	263226_at	AT1G30690	1.59	253878_at	AT4G27550	1.20
247519_at	AT5G61430	3.16	248715_at	AT5G48290	1.58	261401_at	AT1G79640	1.20
261780_at	AT1G76310	3.11	252233_at	AT3G49690	1.58	251488_at	AT3G59440	1.20
261660_at	AT1G18370	3.07	260693_at	AT1G32450	1.58	260417_at	AT1G69770	1.20
267618_at	AT2G26760	3.06	260061_at	AT1G73690	1.58	249011_at	AT5G44670	1.20
254728_at	AT4G13690	3.03	252628_at	AT3G44960	1.58	251256_at	AT3G62300	1.20
245343_at	AT4G15830	3.02	265695_at	AT2G24490	1.58	263920_at	AT2G36410	1.20
248057_at	AT5G55520	2.95	250053_at	AT5G17850	1.58	267619_at	AT2G26730	1.20
265096_at	AT1G04030	2.94	263430_at	AT2G22270	1.57	256832_at	AT3G22880	1.19
252763_at	AT3G42725	2.87	245275_at	AT4G15210	1.57	265453_at	AT2G46520	1.19
259290_at	AT3G11520	2.83	266427_at	AT2G07170	1.57	254490_at	AT4G20320	1.19
267388_at	AT2G44450	2.82	257020_at	AT3G19590	1.56	261080_at	AT1G07370	1.19
250992_at	AT5G02260	2.82	259806_at	AT1G47900	1.56	249970_at	AT5G19100	1.19
246683_at	AT5G33300	2.82	258017_at	AT3G19370	1.56	251660_at	AT3G57160	1.19
253156_at	AT4G35730	2.80	251132_at	AT5G01200	1.56	253132_at	AT4G35580	1.19
255265_at	AT4G05190	2.79	256720_at	AT2G34140	1.56	248456_at	AT5G51380	1.19
247425_at	AT5G62550	2.79	262802_at	AT1G20930	1.56	261924_at	AT1G22550	1.18
264061_at	AT2G27970	2.78	260425_at	AT1G72440	1.56	263047_at	AT2G17630	1.18
259089_at	AT3G04960	2.75	259912_at	AT1G72670	1.55	245604_at	AT4G14290	1.18
245814_at	AT1G49910	2.74	247594_at	AT5G60800	1.55	259228_at	AT3G07720	1.18
263960_at	AT2G36200	2.72	252165_at	AT3G50550	1.55	264340_at	AT1G70280	1.18
259815_at	AT1G49870	2.71	246767_at	AT5G27395	1.55	261761_at	AT1G15660	1.18
265405_at	AT2G16750	2.71	266158_at	AT2G28070	1.55	261211_at	AT1G12780	1.18
258067_at	AT3G25980	2.70	247696_at	AT5G59780	1.55	259518_at	AT1G20510	1.18
257740_at	AT3G27330	2.68	251718_at	AT3G56100	1.54	253364_at	AT4G33160	1.17
257290_at	AT3G15560	2.66	264758_at	AT1G61340	1.54	260011_at	AT1G68110	1.17
251067_at	AT5G01910	2.66	251770_at	AT3G55970	1.54	251538_at	AT3G58660	1.17
265171_at	AT1G23790	2.65	254109_at	AT4G25240	1.53	265383_at	AT2G16780	1.17
255230_at	AT4G05390	2.63	267518_at	AT2G30500	1.53	259076_at	AT3G02140	1.17
250369_at	AT5G11300	2.62	248656_at	AT5G48460	1.53	253975_at	AT4G26600	1.17
254964_at	AT4G11080	2.60	261382_at	AT1G05470	1.53	264895_at	AT1G23100	1.17
255236_at	AT4G05520	2.60	251195_at	AT3G62930	1.52	261741_at	AT1G47870	1.17
263441_at	AT2G28620	2.57	249920_at	AT5G19260	1.52	262943_at	AT1G79470	1.17
266009_at	AT2G37420	2.57	266447_at	AT2G43290	1.52	251418_at	AT3G60440	1.17
252189_at	AT3G50070	2.56	257232_at	AT3G16500	1.52	258529_at	AT3G06740	1.17
246938_at	AT5G25380	2.56	254878_at	AT4G11660	1.52	259908_at	AT1G60850	1.17
254233_at	AT4G23800	2.55	263612_at	AT2G16440	1.52	265326_at	AT2G18220	1.16
251608_at	AT3G57860	2.52	267284_at	AT2G23700	1.52	261832_at	AT1G10650	1.16
248413_at	AT5G51600	2.52	251822_at	AT3G55060	1.51	264357_at	AT1G03360	1.16
256864_at	AT3G23890	2.51	267146_at	AT2G38160	1.51	264111_at	AT2G13690	1.16
247210_at	AT5G65020	2.50	247190_at	AT5G65420	1.51	255080_at	AT4G09030	1.16
247962_at	AT5G56580	2.46	245087_at	AT2G39830	1.50	267339_at	AT2G39870	1.16
264821_at	AT1G03470	2.46	253949_at	AT4G26780	1.50	248021_at	AT5G56500	1.16
259616_at	AT1G47960	2.46	247958_at	AT5G57070	1.50	255094_at	AT4G08590	1.16

257005_at	AT3G14190	2.46	264452_at	AT1G10270	1.50	253407_at	AT4G32920	1.16
255507_at	AT4G02150	2.45	254515_at	AT4G20270	1.50	251556_at	AT3G58840	1.16
264802_at	AT1G08560	2.44	262219_at	AT1G74750	1.50	258998_at	AT3G01820	1.16
252025_at	AT3G52900	2.43	265147_at	AT1G51380	1.49	262571_at	AT1G15430	1.16
251388_at	AT3G60840	2.42	262558_at	AT1G31335	1.49	245617_at	AT4G14490	1.16
264186_at	AT1G54570	2.41	247186_at	AT5G65470	1.49	267082_at	AT2G41140	1.16
257115_at	AT3G20150	2.39	256331_at	AT1G76880	1.49	247310_at	AT5G63950	1.16
248963_at	AT5G45700	2.38	266007_at	AT2G37380	1.49	262713_at	AT1G16520	1.15
245174_at	AT2G47500	2.38	254719_at	AT4G13600	1.48	261586_at	AT1G01640	1.15
255460_at	AT4G02800	2.37	247279_at	AT5G64310	1.48	261178_at	AT1G04760	1.15
250228_at	AT5G13840	2.36	247034_at	AT5G67260	1.48	266363_at	AT2G41250	1.15
250386_at	AT5G11510	2.35	251035_at	AT5G02220	1.47	250087_at	AT5G17270	1.15
249071_at	AT5G44050	2.35	249314_at	AT5G41180	1.47	249354_at	AT5G40480	1.15
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246034_at	AT5G08350	2.34	261387_at	AT1G05410	1.46	264835_at	AT1G03550	1.15
258560_at	AT3G06020	2.33	265935_at	AT2G19580	1.46	257730_at	AT3G18420	1.15
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261085_at	AT1G17480	2.32	248060_at	AT5G55560	1.45	257914_at	AT3G25545	1.15
266655_at	AT2G25880	2.31	266732_at	AT2G03240	1.45	256063_at	AT1G07130	1.14
260144_at	AT1G71960	2.30	257024_at	AT3G19100	1.45	260329_at	AT1G80370	1.14
252148_at	AT3G51280	2.30	257809_at	AT3G27060	1.45	256598_at	AT3G30180	1.14
245302_at	AT4G17695	2.28	264114_at	AT2G31270	1.45	264289_at	AT1G61890	1.14
265085_at	AT1G03780	2.28	262766_at	AT1G13160	1.44	252281_at	AT3G49320	1.14
252938_at	AT4G39190	2.28	248820_at	AT5G47060	1.44	254996_at	AT4G10390	1.14
262109_at	AT1G02730	2.28	251499_at	AT3G59100	1.44	252351_at	AT3G48210	1.14
254349_at	AT4G22250	2.27	265882_at	AT2G42490	1.44	253393_at	AT4G32690	1.14
261917_at	AT1G65920	2.26	259116_at	AT3G01350	1.44	259874_at	AT1G76660	1.13
245202_at	AT1G67720	2.26	262003_at	AT1G64460	1.44	261972_at	AT1G64600	1.13
261859_at	AT1G50490	2.25	265172_at	AT1G23820	1.43	254396_at	AT4G21680	1.13
252077_at	AT3G51720	2.25	267488_at	AT2G19110	1.43	246899_at	AT5G25590	1.13
260438_at	AT1G68290	2.24	266323_at	AT2G46700	1.43	258966_at	AT3G10690	1.13
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245380_at	AT4G17760	2.23	249800_at	AT5G23660	1.42	253614_at	AT4G30350	1.13
251527_at	AT3G58650	2.21	265964_at	AT2G37510	1.42	255225_at	AT4G05410	1.12
251768_at	AT3G55940	2.21	249588_at	AT5G37790	1.41	264219_at	AT1G60420	1.12
253403_at	AT4G32830	2.20	258530_at	AT3G06840	1.41	258454_at	AT3G22300	1.12
245607_at	AT4G14330	2.20	247723_at	AT5G59220	1.41	252785_at	AT3G42660	1.12
257524_at	AT3G01330	2.20	250994_at	AT5G02490	1.41	247748_at	AT5G58920	1.12
261806_at	AT1G30510	2.20	262066_at	AT1G79950	1.41	250234_at	AT5G13420	1.12
262081_at	AT1G59540	2.19	252515_at	AT3G46230	1.41	264790_at	AT2G17820	1.12
266294_at	AT2G29500	2.18	250588_at	AT5G07660	1.41	259220_at	AT3G03550	1.12
249060_at	AT5G44560	2.18	247028_at	AT5G67100	1.40	253249_at	AT4G34680	1.11
253245_at	AT4G34590	2.18	248685_at	AT5G48500	1.40	260824_at	AT1G06720	1.11
258471_at	AT3G06030	2.16	266959_at	AT2G07690	1.39	267305_at	AT2G30070	1.11
257644_at	AT3G25780	2.16	254304_at	AT4G22270	1.39	265154_at	AT1G30960	1.11
246802_at	AT5G27000	2.15	248786_at	AT5G47410	1.39	254455_at	AT4G21140	1.10
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262467_at	AT1G50240	2.14	260869_at	AT1G43800	1.39	253181_at	AT4G35180	1.10
247134_at	AT5G66230	2.13	265943_at	AT2G19570	1.38	260978_at	AT1G53540	1.10
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267006_at	AT2G34190	2.12	266656_at	AT2G25900	1.38	266449_at	AT2G43080	1.10
246995_at	AT5G67470	2.12	256222_at	AT1G56210	1.37	262380_at	AT1G72810	1.10
255790_at	AT2G33560	2.12	247212_at	AT5G65040	1.37	258040_at	AT3G21190	1.10
249555_at	AT5G38300	2.11	260325_at	AT1G63940	1.37	251915_at	AT3G53940	1.10
252089_at	AT3G52110	2.06	264969_at	AT1G67320	1.37	253485_at	AT4G31800	1.10
258480_at	AT3G02640	2.05	260753_at	AT1G49230	1.36	247185_at	AT5G65460	1.09
267529_at	AT2G45490	2.04	256249_at	AT3G11270	1.36	264972_at	AT1G67370	1.09
263535_at	AT2G24970	2.04	247013_at	AT5G67480	1.36	252206_at	AT3G50360	1.09
263017_at	AT2G17620	2.04	255597_at	AT4G01730	1.36	249790_at	AT5G24290	1.09
248579_at	AT5G49880	2.03	257487_at	AT1G71850	1.36	259637_at	AT1G52260	1.09
263383_at	AT2G40120	2.03	247575_at	AT5G61030	1.35	261108_at	AT1G62960	1.09
258859_at	AT3G02120	2.02	250326_at	AT5G12080	1.35	257766_at	AT3G23030	1.09
266989_at	AT2G39330	2.01	251778_at	AT3G55660	1.35	249832_at	AT5G23400	1.09
264763_at	AT1G61450	2.01	264852_at	AT2G17480	1.35	267310_at	AT2G34680	1.09
253051_at	AT4G37490	2.00	254076_at	AT4G25340	1.35	246962_s_at	AT5G24800	1.09
254140_at	AT4G24610	2.00	245063_at	AT2G39795	1.35	259915_at	AT1G72790	1.09
249644_at	AT5G37010	1.99	261309_at	AT1G48600	1.35	259507_at	AT1G43910	1.09

267075_at	AT2G41070	1.99	256013_at	AT1G19270	1.34	256927_at	AT3G22550	1.09
252606_at	AT3G45010	1.99	260328_at	AT1G80360	1.34	249955_at	AT5G18840	1.09
255876_at	AT2G40480	1.98	251147_at	AT3G63480	1.34	260814_at	AT1G43710	1.09
254400_at	AT4G21270	1.97	261607_at	AT1G49660	1.34	251432_at	AT3G59820	1.08
253043_at	AT4G37540	1.97	258377_at	AT3G17690	1.34	259376_at	AT3G16320	1.08
258098_at	AT3G23670	1.97	255757_at	AT4G00460	1.34	253994_at	AT4G26080	1.08
247985_at	AT5G56790	1.97	267358_at	AT2G39890	1.34	256255_at	AT3G11280	1.08
262752_at	AT1G16330	1.97	250937_at	AT5G03230	1.33	247606_at	AT5G61000	1.08
253749_at	AT4G29080	1.96	261415_at	AT1G07750	1.33	254809_at	AT4G12410	1.08
247603_at	AT5G60930	1.96	267123_at	AT2G23560	1.33	263619_at	AT2G04650	1.08
246230_at	AT4G36710	1.96	257610_at	AT3G13810	1.33	259367_at	AT1G69070	1.08
251090_at	AT5G01340	1.95	260349_at	AT1G69400	1.33	251378_at	AT3G60660	1.08
259314_at	AT3G05260	1.94	260387_at	AT1G74100	1.33	252877_at	AT4G39630	1.08
264537_at	AT1G55610	1.94	261551_at	AT1G63440	1.33	247972_at	AT5G56740	1.08
246505_at	AT5G16250	1.94	251923_at	AT3G53880	1.32	262746_at	AT1G28960	1.07
267485_at	AT2G02820	1.94	245259_at	AT4G14150	1.32	253603_at	AT4G30935	1.07
246920_at	AT5G25090	1.93	255410_at	AT4G03100	1.32	257714_at	AT3G27360	1.07
247910_at	AT5G57410	1.93	249997_at	AT5G18620	1.32	257216_at	AT3G14990	1.07
258867_at	AT3G03130	1.93	266322_at	AT2G46690	1.32	246098_at	AT5G20400	1.07
248139_at	AT5G54970	1.93	251680_at	AT3G57060	1.32	266166_at	AT2G28080	1.07
253239_at	AT4G34500	1.93	263882_at	AT2G21790	1.32	253252_at	AT4G34740	1.07
260887_at	AT1G29160	1.92	261100_at	AT1G63020	1.32	247241_at	AT5G64680	1.07
264697_at	AT1G70210	1.92	249068_at	AT5G43980	1.31	264674_at	AT1G09815	1.07
250785_at	AT5G05510	1.91	248341_at	AT5G52220	1.31	267069_at	AT2G41010	1.07
267021_at	AT2G39300	1.91	257086_at	AT3G20490	1.31	267309_at	AT2G19385	1.07
250287_at	AT5G13330	1.91	254379_at	AT4G21820	1.31	249069_at	AT5G44010	1.07
261491_at	AT1G14350	1.91	254274_at	AT4G22770	1.31	265961_at	AT2G37400	1.07
248476_at	AT5G50890	1.90	249728_at	AT5G24390	1.31	255494_at	AT4G02710	1.06
253989_at	AT4G26130	1.90	258385_at	AT3G15510	1.30	260214_at	AT1G74510	1.06
259383_at	AT3G16470	1.89	261713_at	AT1G32640	1.30	259422_at	AT1G13810	1.06
256351_at	AT1G54960	1.89	267127_at	AT2G23610	1.30	264286_at	AT1G61870	1.06
256490_at	AT1G31460	1.89	257502_at	AT1G78110	1.30	250292_at	AT5G13220	1.06
249836_at	AT5G23420	1.89	259686_at	AT1G63100	1.30	250062_at	AT5G17760	1.06
261330_at	AT1G44900	1.88	249916_at	AT5G22880	1.30	262106_at	AT1G02970	1.06
261364_at	AT1G53140	1.88	266297_at	AT2G29570	1.30	249233_at	AT5G42150	1.06
262758_at	AT1G10780	1.87	259303_at	AT3G05130	1.30	249115_at	AT5G43810	1.06
253771_at	AT4G28430	1.85	254079_at	AT4G25730	1.30	257203_at	AT3G23730	1.05
259106_at	AT3G05490	1.84	250814_at	AT5G05080	1.30	257149_at	AT3G27280	1.05
267636_at	AT2G42110	1.83	246095_at	AT5G19310	1.30	250433_at	AT5G10400	1.05
260910_at	AT1G02690	1.83	264377_at	AT2G25060	1.30	251020_at	AT5G02270	1.05
262947_at	AT1G75750	1.83	258202_at	AT3G13940	1.30	264190_at	AT1G54830	1.05
245461_at	AT4G17000	1.83	248708_at	AT5G48560	1.30	250864_at	AT5G03870	1.05
248801_at	AT5G47370	1.81	254810_at	AT4G12390	1.30	264293_at	AT1G78770	1.05
246597_at	AT5G14760	1.81	251332_at	AT3G61670	1.29	247357_at	AT5G63710	1.05
253480_at	AT4G31840	1.81	253175_at	AT4G35050	1.29	261113_at	AT1G75400	1.05
253620_at	AT4G30520	1.80	245849_at	AT5G13520	1.29	261058_at	AT1G01370	1.05
259348_at	AT3G03770	1.79	251109_at	AT5G01600	1.29	262849_at	AT1G14710	1.05
258376_at	AT3G17680	1.79	247009_at	AT5G67600	1.29	253062_at	AT4G37590	1.05
265886_at	AT2G25620	1.79	256675_at	AT3G52170	1.29	253769_at	AT4G28560	1.04
253148_at	AT4G35620	1.77	254990_at	AT4G10610	1.29	247643_at	AT5G60450	1.04
258768_at	AT3G10880	1.77	253777_at	AT4G28450	1.29	256781_at	AT3G13650	1.04
257799_at	AT3G15890	1.76	263955_at	AT2G36010	1.29	251922_at	AT3G54030	1.04
259200_at	AT3G09070	1.76	251760_at	AT3G55605	1.29	255652_at	AT4G00950	1.04
249214_at	AT5G42720	1.75	253058_at	AT4G37660	1.28	258001_at	AT3G28950	1.03
249732_at	AT5G24420	1.75	266812_at	AT2G44830	1.28	265629_at	AT2G27230	1.03
251200_at	AT3G63010	1.75	253592_at	AT4G30840	1.28	258545_at	AT3G07050	1.03
248807_at	AT5G47500	1.75	251029_at	AT5G02050	1.28	259143_at	AT3G10190	1.03
267232_at	AT2G44190	1.74	249372_at	AT5G40760	1.28	249911_at	AT5G22740	1.03
245984_at	AT5G13090	1.74	260157_at	AT1G52930	1.28	258464_at	AT3G17360	1.03
259930_at	AT1G34355	1.73	245364_at	AT4G15790	1.27	258636_at	AT3G07890	1.03
248607_at	AT5G49480	1.73	258877_at	AT3G03220	1.27	251371_at	AT3G60360	1.02
250129_at	AT5G16450	1.72	250844_at	AT5G04470	1.26	247404_at	AT5G62890	1.02
249747_at	AT5G24600	1.72	249027_at	AT5G44790	1.26	247838_at	AT5G57990	1.02
257134_at	AT3G12870	1.72	261077_at	AT1G07430	1.26	259835_at	AT1G52160	1.02
260648_at	AT1G08050	1.72	248674_at	AT5G48800	1.26	257680_at	AT3G20330	1.02
253640_at	AT4G30630	1.72	262704_at	AT1G16530	1.26	261286_at	AT1G35780	1.02
266295_at	AT2G29550	1.72	251037_at	AT5G02100	1.26	258868_at	AT3G03110	1.02

265349_at	AT2G22610	1.71	267034_at	AT2G38310	1.25	261467_at	AT1G28520	1.02
245411_at	AT4G17240	1.71	266227_at	AT2G28870	1.25	247974_at	AT5G56780	1.02
245828_at	AT1G57820	1.70	245422_at	AT4G17470	1.25	259845_at	AT1G73590	1.02
259222_at	AT3G03680	1.70	251206_at	AT3G63090	1.25	259227_at	AT3G07750	1.02
257517_at	AT3G16330	1.69	254933_at	AT4G11130	1.25	258996_at	AT3G01800	1.02
246926_at	AT5G25240	1.68	245339_at	AT4G16120	1.25	261596_at	AT1G33080	1.02
260332_at	AT1G70470	1.68	259513_at	AT1G12430	1.25	251487_at	AT3G59760	1.02
252442_at	AT3G46940	1.68	255874_at	AT2G40550	1.25	255064_at	AT4G08950	1.01
261460_at	AT1G07880	1.68	259862_at	AT1G72650	1.24	253817_at	AT4G28310	1.01
261384_at	AT1G05440	1.68	248286_at	AT5G52870	1.24	249869_at	AT5G23050	1.01
260399_at	AT1G72520	1.67	249838_at	AT5G23460	1.24	253746_at	AT4G29100	1.01
261951_at	AT1G64490	1.67	255530_at	AT4G02140	1.23	250504_at	AT5G09840	1.01
253357_at	AT4G33400	1.67	267118_at	AT2G32590	1.23	258682_at	AT3G08720	1.01
257483_at	AT1G49620	1.67	245879_at	AT5G09420	1.23	256043_at	AT1G07210	1.01
250070_at	AT5G17980	1.65	249266_at	AT5G41670	1.23	250270_at	AT5G12980	1.01
247280_at	AT5G64260	1.65	246346_at	AT3G56810	1.23	248481_at	AT5G50930	1.01
252736_at	AT3G43210	1.64	248658_at	AT5G48600	1.23	261086_at	AT1G17460	1.01
259851_at	AT1G72250	1.64	263039_at	AT1G23280	1.23	247491_at	AT5G61880	1.01
248036_at	AT5G55920	1.00	262193_at	AT1G77840	1.00	251740_at	AT3G56070	1.00
260252_at	AT1G74240	1.00	266510_at	AT2G47990	1.00			

Table S3. Down-regulated transcripts. Genes differentially expressed between DEX treated empty vector control and *IOE-OBP1* plants. Genes were considered to exhibit an altered expression level when the hybridization signal was >2-fold decreased in comparison between the two sets of plants.

AffyID	Locus	Change (log_2)	AffyID	Locus	Change (log_2)	AffyID	Locus	Change (log_2)
260221_at	AT1G74670	-4.03	255856_at	AT1G66940	-1.59	256825_at	AT3G22120	-1.24
255822_at	AT2G40610	-3.63	262830_at	AT1G14700	-1.58	261346_at	AT1G79720	-1.24
264774_at	AT1G22890	-3.45	266275_at	AT2G29370	-1.58	258993_at	AT3G08940	-1.24
249346_at	AT5G40780	-3.40	257300_at	AT3G28080	-1.58	255148_at	AT4G08470	-1.24
245038_at	AT2G26560	-3.37	255692_at	AT4G00400	-1.58	265893_at	AT2G15040	-1.24
253971_at	AT4G26530	-3.27	258528_at	AT3G06770	-1.58	249008_at	AT5G44680	-1.24
252549_at	AT3G45860	-3.25	261230_at	AT1G20010	-1.58	261318_at	AT1G53035	-1.24
251746_at	AT3G56060	-3.23	266215_at	AT2G06850	-1.58	257966_at	AT3G19800	-1.24
267546_at	AT2G32680	-3.20	253278_at	AT4G34220	-1.58	254688_at	AT4G13830	-1.24
265471_at	AT2G37130	-3.12	247602_at	AT5G60900	-1.58	248335_at	AT5G52450	-1.24
257964_at	AT3G19850	-3.11	262878_at	AT1G64770	-1.58	247440_at	AT5G62680	-1.24
254247_at	AT4G23260	-3.06	263202_at	AT1G05630	-1.58	257745_at	AT3G29240	-1.23
252317_at	AT3G48720	-3.04	260837_at	AT1G43670	-1.57	259430_at	AT1G01610	-1.23
247684_at	AT5G59670	-3.02	256874_at	AT3G26320	-1.57	250734_at	AT5G06270	-1.23
256787_at	AT3G13790	-2.93	251183_at	AT3G62630	-1.57	254133_at	AT4G24810	-1.23
261449_at	AT1G21120	-2.92	252234_at	AT3G49780	-1.57	254746_at	AT4G12980	-1.23
253305_at	AT4G33666	-2.89	248124_at	AT5G54730	-1.57	258897_at	AT3G05730	-1.23
254564_at	AT4G19170	-2.89	262286_at	AT1G68585	-1.57	265539_at	AT2G15830	-1.23
256299_at	AT1G69530	-2.82	249810_at	AT5G23920	-1.57	254256_at	AT4G23180	-1.23
258537_at	AT3G04210	-2.80	266037_at	AT2G05940	-1.57	248763_at	AT5G47550	-1.23
246603_at	AT1G31690	-2.75	257805_at	AT3G18830	-1.56	263664_at	AT1G04250	-1.23
249125_at	AT5G43450	-2.73	259910_at	AT1G72700	-1.56	266873_at	AT2G44740	-1.23
247252_at	AT5G64770	-2.72	263777_at	AT2G46450	-1.56	255889_at	AT1G17840	-1.23
245304_at	AT4G15630	-2.71	255340_at	AT4G04490	-1.56	249128_at	AT5G43440	-1.23
258468_at	AT3G06070	-2.71	250752_at	AT5G05690	-1.56	264313_at	AT1G70410	-1.22
265066_at	AT1G03870	-2.69	254040_at	AT4G25900	-1.56	263184_at	AT1G05560	-1.22
245265_at	AT4G14400	-2.67	260411_at	AT1G69890	-1.56	256766_at	AT3G22231	-1.22
252076_at	AT3G51660	-2.64	263106_at	AT2G05160	-1.55	254573_at	AT4G19420	-1.22
255016_at	AT4G10120	-2.64	261420_at	AT1G07720	-1.55	263473_at	AT2G31750	-1.22
266385_at	AT2G14610	-2.63	266583_at	AT2G46220	-1.55	245176_at	AT2G47440	-1.22
262793_at	AT1G13110	-2.63	257635_at	AT3G26280	-1.55	256455_at	AT1G75190	-1.22
248932_at	AT5G46050	-2.62	261363_at	AT1G41830	-1.55	263495_at	AT2G42530	-1.22
257624_at	AT3G26220	-2.60	257625_at	AT3G26230	-1.55	248353_at	AT5G52320	-1.22
263595_at	AT2G01890	-2.59	251916_at	AT3G53960	-1.55	254333_at	AT4G22753	-1.21
251625_at	AT3G57260	-2.59	249472_at	AT5G39210	-1.55	267084_at	AT2G41180	-1.21
247314_at	AT5G64000	-2.57	257697_at	AT3G12700	-1.55	264201_at	AT1G22630	-1.21
258764_at	AT3G10720	-2.55	245046_at	AT2G26510	-1.54	264609_at	AT1G04530	-1.21
256021_at	AT1G58270	-2.55	247072_at	AT5G66490	-1.54	261788_at	AT1G15980	-1.21
258919_at	AT3G10525	-2.55	267592_at	AT2G39710	-1.53	246313_at	AT1G31920	-1.21

259925_at	AT1G75040	-2.54	255502_at	AT4G02410	-1.53	246947_at	AT5G25120	-1.21
252983_at	AT4G37980	-2.53	245346_at	AT4G17090	-1.53	253779_at	AT4G28490	-1.20
249941_at	AT5G22270	-2.52	257297_at	AT3G28040	-1.53	245876_at	AT1G26230	-1.20
245117_at	AT2G41560	-2.49	255690_at	AT4G00360	-1.53	264507_at	AT1G09415	-1.20
247162_at	AT5G65730	-2.49	249486_at	AT5G39030	-1.53	260070_at	AT1G73830	-1.20
265342_at	AT2G18300	-2.46	263715_at	AT2G20570	-1.53	262883_at	AT1G64780	-1.20
267567_at	AT2G30770	-2.46	266658_at	AT2G25735	-1.53	263799_at	AT2G24550	-1.20
258552_at	AT3G07010	-2.45	256754_at	AT3G25690	-1.53	257925_at	AT3G23170	-1.20
255524_at	AT4G02330	-2.45	260531_at	AT2G47240	-1.53	258023_at	AT3G19450	-1.20
252972_at	AT4G38840	-2.44	256304_at	AT1G69523	-1.52	248246_at	AT5G53200	-1.20
259789_at	AT1G29395	-2.44	260287_at	AT1G80440	-1.52	260388_at	AT1G74070	-1.20
246573_at	AT1G31680	-2.43	246700_at	AT5G28030	-1.52	259560_at	AT1G21270	-1.19
256237_at	AT3G12610	-2.43	265894_at	AT2G15050	-1.52	253608_at	AT4G30290	-1.19
253088_at	AT4G36220	-2.42	255549_at	AT4G01950	-1.52	258013_at	AT3G19320	-1.19
258920_at	AT3G10520	-2.40	266089_at	AT2G38010	-1.51	253083_at	AT4G36250	-1.19
245463_at	AT4G17030	-2.40	259443_at	AT1G02360	-1.51	251299_at	AT3G61950	-1.19
257769_at	AT3G23050	-2.40	263987_at	AT2G42690	-1.51	265414_at	AT2G16660	-1.19
256469_at	AT1G32540	-2.40	259660_at	AT1G55260	-1.50	248719_at	AT5G47910	-1.19
245264_at	AT4G17245	-2.39	252367_at	AT3G48360	-1.50	253421_at	AT4G32340	-1.19
267209_at	AT2G30930	-2.39	253302_at	AT4G33660	-1.50	250503_at	AT5G09820	-1.19
254975_at	AT4G10500	-2.38	251443_at	AT3G59940	-1.50	262414_at	AT1G49430	-1.19
246272_at	AT4G37150	-2.37	249004_at	AT5G44570	-1.50	258792_at	AT3G04640	-1.19
251575_at	AT3G58120	-2.37	247478_at	AT5G62360	-1.49	250498_at	AT5G09660	-1.18
266983_at	AT2G39400	-2.37	247327_at	AT5G64120	-1.49	246293_at	AT3G56710	-1.18
259375_at	AT3G16370	-2.34	266745_at	AT2G02950	-1.49	260419_at	AT1G69730	-1.18
247356_at	AT5G63800	-2.33	248551_at	AT5G50200	-1.49	259000_at	AT3G01860	-1.18
248028_at	AT5G55620	-2.33	261431_at	AT1G18710	-1.49	256593_at	AT3G28510	-1.18
250936_at	AT5G03120	-2.33	257061_at	AT3G18250	-1.49	249515_at	AT5G38530	-1.18
246781_at	AT5G27350	-2.32	246195_at	AT4G36410	-1.49	262254_at	AT1G53920	-1.18
261339_at	AT1G35710	-2.32	267158_at	AT2G37640	-1.49	255774_at	AT1G18620	-1.18
266259_at	AT2G27830	-2.32	266979_at	AT2G39470	-1.49	248623_at	AT5G49170	-1.18
248794_at	AT5G47220	-2.30	248118_at	AT5G55050	-1.48	259058_at	AT3G03470	-1.18
261226_at	AT1G20190	-2.28	267357_at	AT2G40000	-1.48	250028_at	AT5G18130	-1.17
249775_at	AT5G24160	-2.28	253161_at	AT4G35770	-1.48	266899_at	AT2G34620	-1.17
256914_at	AT3G23880	-2.28	259163_at	AT3G01490	-1.48	254356_at	AT4G22190	-1.17
246550_at	AT5G14920	-2.27	251189_at	AT3G62650	-1.48	252971_at	AT4G38770	-1.17
250968_at	AT5G02890	-2.27	260005_at	AT1G67920	-1.48	252441_at	AT3G46780	-1.17
260924_at	AT1G21590	-2.27	254252_at	AT4G23310	-1.48	259892_at	AT1G72610	-1.17
255607_at	AT4G01130	-2.26	263478_at	AT2G31880	-1.47	248756_at	AT5G47560	-1.17
257008_at	AT3G14210	-2.26	257474_at	AT1G80850	-1.47	266927_at	AT2G45960	-1.17
267264_at	AT2G22970	-2.26	254239_at	AT4G23400	-1.47	246755_at	AT5G27920	-1.17
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262049_at	AT1G80180	-1.69	252322_at	AT3G48550	-1.28	260254_at	AT1G74210	-1.03
254271_at	AT4G23150	-1.69	256060_at	AT1G07050	-1.28	259985_at	AT1G76620	-1.03
264672_at	AT1G09750	-1.69	263948_at	AT2G35980	-1.28	255411_at	AT4G03110	-1.03
258956_at	AT3G01440	-1.69	248975_at	AT5G45040	-1.28	255008_at	AT4G10060	-1.03
259502_at	AT1G15670	-1.68	258250_at	AT3G15850	-1.28	259751_at	AT1G71030	-1.03
253943_at	AT4G27030	-1.68	252573_at	AT3G45260	-1.28	248568_at	AT5G49760	-1.03
252411_at	AT3G47430	-1.68	256516_at	AT1G66150	-1.28	262455_at	AT1G11310	-1.03
251330_at	AT3G61550	-1.67	248912_at	AT5G45670	-1.28	266139_at	AT2G28085	-1.03
253052_at	AT4G37310	-1.67	260696_at	AT1G32520	-1.28	264779_at	AT1G08570	-1.03
254032_at	AT4G25940	-1.67	266483_at	AT2G47910	-1.28	254487_at	AT4G20780	-1.02
245637_at	AT1G25230	-1.67	255127_at	AT4G08300	-1.28	263931_at	AT2G36220	-1.02
259783_at	AT1G29510	-1.67	264444_at	AT1G27360	-1.28	256300_at	AT1G69490	-1.02
265400_at	AT2G10940	-1.67	262899_at	AT1G59870	-1.28	266017_at	AT2G18690	-1.02
259308_at	AT3G05180	-1.67	260856_at	AT1G21910	-1.28	260037_at	AT1G68840	-1.02
258894_at	AT3G05650	-1.67	248303_at	AT5G53170	-1.27	266123_at	AT2G45180	-1.02
257701_at	AT3G12710	-1.66	266552_at	AT2G46330	-1.27	245731_at	AT1G73500	-1.02
266790_at	AT2G28950	-1.65	250265_at	AT5G12900	-1.27	246596_at	AT5G14740	-1.02
248839_at	AT5G46690	-1.65	252437_at	AT3G47380	-1.27	250892_at	AT5G03760	-1.02
252563_at	AT3G45970	-1.65	256933_at	AT3G22600	-1.27	251073_at	AT5G01750	-1.02
246998_at	AT5G67370	-1.65	248606_at	AT5G49450	-1.27	253038_at	AT4G37790	-1.02
255294_at	AT4G04750	-1.65	257206_at	AT3G16530	-1.27	245701_at	AT5G04140	-1.02
254553_at	AT4G19530	-1.64	257734_at	AT3G18370	-1.27	252214_at	AT3G50260	-1.02

267199_at	AT2G30990	-1.64	259398_at	AT1G17700	-1.27	261785_at	AT1G08230	-1.02
262651_at	AT1G14100	-1.64	266707_at	AT2G03310	-1.27	252419_at	AT3G47510	-1.01
260551_at	AT2G43510	-1.64	254119_at	AT4G24780	-1.27	247812_at	AT5G58390	-1.01
254245_at	AT4G23240	-1.63	247903_at	AT5G57340	-1.27	251658_at	AT3G57020	-1.01
246701_at	AT5G28020	-1.63	258708_at	AT3G09580	-1.27	262539_at	AT1G17200	-1.01
267517_at	AT2G30520	-1.63	253956_at	AT4G26700	-1.26	261378_at	AT1G18890	-1.01
255596_at	AT4G01720	-1.63	258857_at	AT3G02110	-1.26	250007_at	AT5G18670	-1.01
246238_at	AT4G36670	-1.62	258350_at	AT3G17510	-1.26	253387_at	AT4G33010	-1.01
253722_at	AT4G29190	-1.62	254122_at	AT4G24510	-1.26	262134_at	AT1G77990	-1.01
264901_at	AT1G23090	-1.62	250074_at	AT5G17310	-1.26	265025_at	AT1G24575	-1.01
258472_at	AT3G06080	-1.62	257636_at	AT3G26200	-1.26	245943_at	AT5G19500	-1.01
259996_at	AT1G67910	-1.62	247943_at	AT5G57170	-1.26	247450_at	AT5G62350	-1.01
255662_at	AT4G00400	-1.62	257868_at	AT3G25070	-1.26	258434_at	AT3G16770	-1.00
250613_at	AT5G07240	-1.62	250079_at	AT5G16650	-1.26	264653_at	AT1G08980	-1.00
259841_at	AT1G52200	-1.62	252976_sat	AT4G38550	-1.26	254416_at	AT4G21380	-1.00
248683_at	AT5G48490	-1.61	256676_at	AT3G52180	-1.26	254370_at	AT4G21750	-1.00
258181_at	AT3G21670	-1.61	249581_at	AT5G37600	-1.26			
264371_at	AT1G12090	-1.60	259340_at	AT3G03870	-1.25			
252534_at	AT3G46130	-1.60	256880_at	AT3G26450	-1.25			
264195_at	AT1G22690	-1.60	258094_at	AT3G14690	-1.25			
264898_at	AT1G23205	-1.59	256796_at	AT3G22210	-1.25			
252199_at	AT3G50270	-1.59	250818_at	AT5G04930	-1.25			
266265_at	AT2G29340	-1.59	264866_at	AT1G24140	-1.25			
252543_at	AT3G45780	-1.59	267385_at	AT2G44380	-1.25			
250045_at	AT5G17700	-1.59	262745_at	AT1G28600	-1.24			
248686_at	AT5G48540	-1.59	263549_at	AT2G21650	-1.24			

Table S4. Overlap between genes affected in *IOE-OBP1* plants and genes that expression was shown to oscillate during cell cycle in aphidicolin block/release experiments (Menges *et al.,* 2003).

AffyID	Description	locus	Change (log_2)	Cell cycle phase
252210_at	OBP1 (OBF BINDING PROTEIN 1); DNA binding / transcription factor	AT3G50410	5.42	G1
252691_at	kinesin motor protein-related	AT3G44050	4.01	M
246415_at	unknown protein	AT5G17160	3.83	M
249809_at	microtubule motor	AT5G23910	3.43	M
248691_at	unknown protein	AT5G48310	3.40	M
253978_at	unknown protein	AT4G26660	3.38	M
246557_at	unknown protein	AT5G15510	3.35	M
250823_at	unknown protein	AT5G05180	3.26	M
253804_at	unknown protein	AT4G28230	3.25	M
259978_at	CDKB2;1 (CYCLIN-DEPENDENT KINASE B2;1); kinase	AT1G76540	3.21	M
247039_at	ATEB1C (MICROTUBULE END BINDING PROTEIN 1); microtubule binding	AT5G67270	3.21	M
261780_at	CYCB2;4 (CYCLIN B2;4); cyclin-dependent protein kinase regulator	AT1G76310	3.11	M
261660_at	HIK (HINKEL); microtubule motor	AT1G18370	3.07	M
267618_at	CYCB1;4 (CYCLIN 3); cyclin-dependent protein kinase regulator	AT2G26760	3.06	M
245343_at	binding	AT4G15830	3.02	M
248057_at	unknown protein	AT5G55520	2.95	M
265096_at	unknown protein	AT1G04030	2.94	M
252763_at	unknown protein	AT3G42725	2.87	M
259290_at	CYCB1;3 (CYCLIN B1;3); cyclin-dependent protein kinase regulator	AT3G11520	2.83	M
253156_at	unknown protein	AT4G35730	2.80	M
255265_at	ATK5 (Arabidopsis thaliana kinesin 5); microtubule motor	AT4G05190	2.79	M
247425_at	unknown protein	AT5G62550	2.79	M
259815_at	unknown protein	AT1G49870	2.71	M
258067_at	mitotic spindle checkpoint protein, putative (MAD2)	AT3G25980	2.70	M
257740_at	zinc finger (C3HC4-type RING finger) family protein	AT3G27330	2.68	M
257290_at	unknown protein	AT3G15560	2.66	M
251067_at	unknown protein	AT5G01910	2.66	M
265171_at	unknown protein	AT1G23790	2.65	M
250369_at	CYC3B (MITOTIC-LIKE CYCLIN 3B FROM ARABIDOPSIS)	AT5G11300	2.62	M
254964_at	high mobility group (HMG1/2) family protein	AT4G11080	2.60	M
255236_at	calcium-binding EF hand family protein	AT4G05520	2.60	M
263441_at	kinesin motor protein-related	AT2G28620	2.57	M
266009_at	kinesin motor protein-related	AT2G37420	2.57	M

246938_at	CYCA2;1 (CYCLIN A2;1); cyclin-dependent protein kinase regulator	AT5G25380	2.56	M
254233_at	high mobility group (HMG1/2) family protein	AT4G23800	2.55	M
248413_at	PLE (PLEIADE)	AT5G51600	2.52	M
256864_at	TOPII (TOPOISOMERASE II)	AT3G23890	2.51	M
247210_at	ANNAT2 (ANNEXIN ARABIDOPSIS 2)	AT5G65020	2.50	S
257005_at	similar to unknown protein	AT3G14190	2.46	M
255507_at	ATIMPALPHA3/MOS6; protein transporter	AT4G02150	2.45	M
264802_at	SYP111 (syntaxin 111); t-SNARE	AT1G08560	2.44	M
251388_at	microtubule associated protein (MAP65/ASE1) family protein	AT3G60840	2.42	M
257115_at	kinesin motor family protein	AT3G20150	2.39	M
248963_at	NLI interacting factor (NIF) family protein	AT5G45700	2.38	M
245174_at	microtubule motor	AT2G47500	2.38	M
255460_at	unknown protein	AT4G02800	2.37	M
250228_at	WD-40 repeat family protein	AT5G13840	2.36	M
250386_at	MYB3R-4transcription factor	AT5G11510	2.35	M
258560_at	unknown protein	AT3G06020	2.33	M
266655_at	ATAUR2 (ATAURORA2); histone serine kinase(H3-S10 specific) / kinase	AT2G25880	2.31	M
252148_at	male sterility MS5, putative	AT3G51280	2.30	M
265085_at	targeting protein-related	AT1G03780	2.28	M
262109_at	ATCSLD5 (Cellulose synthase-like D5); cellulose synthase	AT1G02730	2.28	M
261859_at	UBC20 (ubiquitin-conjugating enzyme 20); ubiquitin-protein ligase	AT1G50490	2.25	M
252077_at	unknown protein	AT3G51720	2.25	M
260438_at	bifunctional nuclease, putative	AT1G68290	2.24	G1
245348_at	ATTPS5 (Arabidopsis thaliana trehalose phosphatase/synthase 5)	AT4G17770	2.24	M
245380_at	damaged DNA binding / exonuclease	AT4G17760	2.23	S
251527_at	unknown protein	AT3G58650	2.21	M
253403_at	ATAUR1 (ATAURORA1)	AT4G32830	2.20	M
245607_at	phragmoplast-associated kinesin-related protein 2 (PAKRP2)	AT4G14330	2.20	M
262081_at	ZCF125; microtubule motor	AT1G59540	2.19	M
266294_at	17.6 kDa class I small heat shock protein (HSP17.6B-CI)	AT2G29500	2.18	S
249060_at	SNF7 family protein	AT5G44560	2.18	M
245739_at	CYCA1;1 (CYCLIN A1;1); cyclin-dependent protein kinase regulator	AT1G44110	2.15	M
247134_at	unknown protein	AT5G66230	2.13	M
252078_at	IMK2 (INFLORESCENCE MERISTEM RECEPTOR-LIKE KINASE 2)	AT3G51740	2.12	M
255790_at	spindle checkpoint protein-related	AT2G33560	2.12	M
249555_at	unknown protein	AT5G38300	2.11	M
252089_at	unknown protein	AT3G52110	2.06	M
258480_at	unknown protein	AT3G02640	2.05	M
263017_at	CYCB2;1 (CYCLIN B2;1); cyclin-dependent protein kinase regulator	AT2G17620	2.04	M
258859_at	hydroxyproline-rich glycoprotein family protein	AT3G02120	2.02	M
264763_at	unknown protein	AT1G61450	2.01	M
249644_at	unknown protein	AT5G37010	1.99	M
255876_at	unknown protein	AT2G40480	1.98	G1
258098_at	PAKRP1L; microtubule motor	AT3G23670	1.97	M
262752_at	CYCB3;1 (CYCLIN B3;1); cyclin-dependent protein kinase	AT1G16330	1.97	M
247603_at	chromosome-associated kinesin, putative	AT5G60930	1.96	M
246505_at	unknown protein	AT5G16250	1.94	M
267485_at	MYB88 (myb domain protein 88); DNA binding / transcription factor	AT2G02820	1.94	G2
246920_at	plastocyanin-like domain-containing protein	AT5G25090	1.93	M
258867_at	unknown protein	AT3G03130	1.93	M
250785_at	protein kinase-related	AT5G05510	1.91	M
248476_at	unknown protein	AT5G50890	1.90	M
256490_at	unknown protein	AT1G31460	1.89	M
249836_at	HMGB6 (High mobility group B 6); transcription factor	AT5G23420	1.89	S
261364_at	dynamin family protein	AT1G53140	1.88	M
262758_at	F-box family protein	AT1G10780	1.87	M
253771_at	reticulon family protein	AT4G28430	1.85	M
259106_at	RALFL22 (RALF-LIKE 22)	AT3G05490	1.84	M
267636_at	unknown protein	AT2G42110	1.83	M
260910_at	importin alpha-2 subunit, putative	AT1G02690	1.83	M
245461_at	unknown protein	AT4G17000	1.83	M
246597_at	AO (L-ASPARTATE OXIDASE); L-aspartate oxidase	AT5G14760	1.81	G2
253480_at	plastocyanin-like domain-containing protein	AT4G31840	1.81	M
258376_at	unknown protein	AT3G17680	1.79	M
253148_at	CYCB2;2 (CYCLIN B2;2); cyclin-dependent protein kinase regulator	AT4G35620	1.77	M
258768_at	unknown protein	AT3G10880	1.77	G2
248807_at	pectinesterase family protein	AT5G47500	1.75	M

267232_at	unknown protein	AT2G44190	1.74	M
259930_at	forkhead-associated domain-containing protein	AT1G34355	1.73	M
257134_at	unknown protein	AT3G12870	1.72	M
265349_at	kinesin motor protein-related	AT2G22610	1.71	M
259222_at	C2 domain-containing protein	AT3G03680	1.70	M
246926_at	similar to NHL repeat-containing protein	AT5G25240	1.68	M
260332_at	unknown protein	AT1G70470	1.68	M
252442_at	deoxyuridine 5'-triphosphate nucleotidohydrolase family	AT3G46940	1.68	S
261460_at	ATMPK13 (ARABIDOPSIS THALIANA MAP KINASE 13); MAP kinase	AT1G07880	1.68	M
261384_at	similar to Os03g0747100	AT1G05440	1.68	M
253357_at	dem protein-related / defective embryo and meristems protein-related	AT4G33400	1.67	M
250070_at	C2 domain-containing protein	AT5G17980	1.65	G1
259851_at	kinesin motor protein-related	AT1G72250	1.64	M
251281_at	AGP20 (ARABINOGALACTAN PROTEIN 20)	AT3G61640	1.62	G1
248055_at	lectin protein kinase, putative	AT5G55830	1.60	M
263226_at	SEC14 cytosolic factor family protein / phosphoglyceride transfer	AT1G30690	1.59	M
265695_at	ATRPA2/ROR1/RPA2 (REPLICON PROTEIN A); protein binding	AT2G24490	1.58	S
266427_at	binding	AT2G07170	1.57	M
257020_at	WD-40 repeat family protein / mitotic checkpoint protein, putative	AT3G19590	1.56	M
256720_at	Dof-type zinc finger domain-containing protein	AT2G34140	1.56	S
262802_at	CDKB2;2 (CYCLIN-DEPENDENT KINASE B2;2); kinase	AT1G20930	1.56	M
254109_at	SKS1 (SKU5 SIMILAR 1); copper ion binding	AT4G25240	1.53	M
248656_at	fimbrin-like protein, putative	AT5G48460	1.53	M
249920_at	unknown protein	AT5G19260	1.52	G1
266447_at	MSS3	AT2G43290	1.52	G1
251822_at	unknown protein)	AT3G55060	1.51	M
267146_at	similar to proline-rich family protein	AT2G38160	1.51	M
247190_at	CYCD4;1 (CYCLIN D4;1); cyclin-dependent protein kinase regulator	AT5G65420	1.51	S
262558_at	similar to Os03g0780200	AT1G31335	1.49	M
250756_at	ATROPGEF5/ROPGEF5	AT5G05940	1.46	M
257024_at	calcium-dependent protein kinase, putative / CDPK, putative	AT3G19100	1.45	G2
264114_at	ATCDT1A/CDT1/CDT1A	AT2G31270	1.45	M
253203_at	ADC2 (ARGININE DECARBOXYLASE 2)	AT4G34710	1.42	G2
249588_at	protein kinase family protein	AT5G37790	1.41	S
247723_at	protein phosphatase 2C, putative / PP2C, putative	AT5G59220	1.41	G2
252515_at	ATHSP17.4 (Arabidopsis thaliana heat shock protein 17.4)	AT3G46230	1.41	S
266959_at	minichromosome maintenance family protein / MCM family protein	AT2G07690	1.39	G1
246776_at	kinesin motor protein-related	AT5G27550	1.39	M
260869_at	acyl-(acyl-carrier-protein) desaturase, putative	AT1G43800	1.39	S
247013_at	BT4 (BTB AND TAZ DOMAIN PROTEIN 4)	AT5G67480	1.36	G2
255597_at	zinc finger (DHHC type) family protein	AT4G01730	1.36	M
251778_at	ATROPGEF6/ROPGEF6 (KINASE PARTNER PROTEIN-LIKE)	AT3G55660	1.35	M
261607_at	similar to cell death associated protein-related	AT1G49660	1.34	G1
260387_at	sulfotransferase family protein	AT1G74100	1.33	S
255410_at	rac GTPase activating protein, putative	AT4G03100	1.32	G2
248341_at	similar to hypothetical protein	AT5G52220	1.31	S
254379_at	calmodulin-binding family protein	AT4G21820	1.31	M
259686_at	scarecrow transcription factor family protein	AT1G63100	1.30	M
249916_at	histone H2B, putative	AT5G22880	1.30	S
264377_at	plastocyanin-like domain-containing protein	AT2G25060	1.30	M
254810_at	invertase/pectin methylesterase inhibitor family protein	AT4G12390	1.30	G1
251109_at	ATFER1 (ferretin 1); ferric iron binding	AT5G01600	1.29	M
256675_at	DNA binding	AT3G52170	1.29	M
266812_at	protein kinase, putative	AT2G44830	1.28	S
249372_at	G6PD6 (GLUCOSE-6-PHOSPHATE DEHYDROGENASE 6)	AT5G40760	1.28	S
248674_at	phototropic-responsive NPH3 family protein	AT5G48800	1.26	G1
255874_at	similar to Os01g0166800	AT2G40550	1.25	G1
248658_at	ATSMC3	AT5G48600	1.23	S
258609_at	Identical to UPF0131 protein At3g02910	AT3G02910	1.21	S
249011_at	unknown protein	AT5G44670	1.20	G1
254490_at	CTP synthase	AT4G20320	1.19	G1
251660_at	unknown protein	AT3G57160	1.19	G1
261761_at	similar to CENP-C [Medicago truncatula]	AT1G15660	1.18	S
258529_at	zinc finger (GATA type) family protein	AT3G06740	1.17	S
261832_at	protein binding / zinc ion binding	AT1G10650	1.16	G1
255080_at	AGP10 (Arabinogalactan protein 10)	AT4G09030	1.16	M
245617_at	forkhead-associated domain-containing protein	AT4G14490	1.16	M

254396_at	proton-dependent oligopeptide transport (POT) family protein	AT4G21680	1.13	G2
246899_at	unknown protein	AT5G25590	1.13	M
264790_at	ATHK1 (HISTIDINE KINASE 1)	AT2G17820	1.12	G1
253181_at	LHT7 (LYS/HIS TRANSPORTER 7); amino acid permease	AT4G35180	1.10	G2
260978_at	17.6 kDa class I small heat shock protein (HSP17.6C-CI) (AA 1-156)	AT1G53540	1.10	S
257467_at	LOB domain protein 4 / lateral organ boundaries domain protein 4 (LBD4)	AT1G31320	1.10	G1
257766_at	IAA2 (indoleacetic acid-induced protein 2); transcription factor	AT3G23030	1.09	G2
249832_at	disease resistance family protein / LRR family protein	AT5G23400	1.09	M
247606_at	replication protein, putative	AT5G61000	1.08	S
252877_at	similar to Os08g0290400	AT4G39630	1.08	S
257714_at	histone H3	AT3G27360	1.07	S
257216_at	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein	AT3G14990	1.07	G2
255494_at	kinase interacting family protein	AT4G02710	1.06	G1
250292_at	unknown protein	AT5G13220	1.06	M
262106_at	WEE1 (Arabidopsis wee1 kinase homolog); kinase/ protein kinase	AT1G02970	1.06	S
249115_at	ZLL (ZWILLE)	AT5G43810	1.06	G2
250864_at	glutaredoxin family protein	AT5G03870	1.05	M
261058_at	HTR12 (Centromeric histone H3); DNA binding	AT1G01370	1.05	S
265629_at	transcription factor-related	AT2G27230	1.03	G2
249911_at	ATCSLA02 (Cellulose synthase-like A2)	AT5G22740	1.03	G1
258464_at	POK1 (PHRAGMOPLAST ORIENTING KINESIN 1); microtubule motor	AT3G17360	1.03	M
259845_at	PIN1 (PIN-FORMED 1); transporter	AT1G73590	1.02	G2
253817_at	unknown protein	AT4G28310	1.01	S
249869_at	acyl-activating enzyme 17 (AAE17)	AT5G23050	1.01	G2
254416_at	ARK3 (Arabidopsis Receptor Kinase 3); kinase	AT4G21380	-1.00	S
247450_at	invertase/pectin methylesterase inhibitor family protein	AT5G62350	-1.01	G1
249527_at	proline oxidase, putative	AT5G38710	-1.03	G1
262262_at	unknown protein	AT1G70780	-1.04	S
248200_at	ATOMT1 (O-METHYLTRANSFERASE 1)	AT5G54160	-1.04	S
263419_at	protein kinase, putative	AT2G17220	-1.06	S
262230_at	ATXYL1/XYL1 (ALPHA-XYLOSIDASE 1	AT1G68560	-1.06	G1
262643_at	invertase/pectin methylesterase inhibitor family protein	AT1G62770	-1.07	G1
246488_at	3-oxo-5-alpha-steroid 4-dehydrogenase family protein	AT5G16010	-1.08	S
256366_at	serine/threonine protein kinase family protein	AT1G66880	-1.10	S
247671_at	cytoplasmic linker protein-related	AT5G60210	-1.12	M
249876_at	unknown protein	AT5G23060	-1.16	G1
245329_at	zinc finger (C3HC4-type RING finger) family protein	AT4G14365	-1.17	S
262254_at	GLIP5 (GDSL-motif lipase 5); carboxylic ester hydrolase	AT1G53920	-1.18	S
246293_at	SIB1 (SIGMA FACTOR BINDING PROTEIN 1); binding	AT3G56710	-1.18	M
265414_at	nodulin family protein	AT2G16660	-1.19	G1
253608_at	ATXTH19	AT4G30290	-1.19	S
254333_at	SMO1-3 (STEROL 4-ALPHA METHYL OXIDASE); catalytic	AT4G22753	-1.21	G1
263664_at	AXR3 (AUXIN RESISTANT 3); transcription factor	AT1G04250	-1.23	M
254119_at	pectate lyase family protein	AT4G24780	-1.27	G1
260856_at	AP2 domain-containing transcription factor family protein	AT1G21910	-1.28	S
254608_at	NIP1;2/NLM2 (NOD26-like intrinsic protein 1;2); water channel	AT4G18910	-1.31	S
259736_at	endo-1,4-beta-glucanase, putative / cellulase, putative	AT1G64390	-1.32	G1
249393_at	disease resistance family protein	AT5G40170	-1.32	S
261266_at	ATEXPA10 (ARABIDOPSIS THALIANA EXPANSIN A10)	AT1G26770	-1.32	G1
265648_at	glycosyl hydrolase family 17 protein	AT2G27500	-1.34	G1
266461_at	ATGSTF8 (GLUTATHIONE S-TRANSFERASE 8); glutathione transferase	AT2G47730	-1.36	S
258641_at	unknown protein	AT3G08030	-1.37	G1
267367_at	unknown protein	AT2G44210	-1.39	S
248646_at	similar to OJ000315_02.19	AT5G49100	-1.39	G1
246884_at	ChaC-like family protein	AT5G26220	-1.41	G1
266299_at	ATGSTU5	AT2G29450	-1.41	G1
254221_at	glycoside hydrolase family 28 protein	AT4G23820	-1.42	M
248327_at	heavy-metal-associated domain-containing protein	AT5G52750	-1.42	S
263073_at	auxin efflux carrier family protein	AT2G17500	-1.45	S
252863_at	MI-1-P SYNTHASE (Myo-inositol-1-phosphate synthase)	AT4G39800	-1.47	G1
263478_at	leucine-rich repeat transmembrane protein kinase, putative	AT2G31880	-1.47	S
257297_at	leucine-rich repeat transmembrane protein kinase, putative	AT3G28040	-1.53	M
253278_at	leucine-rich repeat transmembrane protein kinase, putative	AT4G34220	-1.58	G1
252563_at	ATEXLA1 (ARABIDOPSIS THALIANA EXPANSIN-LIKE A1)	AT3G45970	-1.65	S
266790_at	ATEXPA6 (ARABIDOPSIS THALIANA EXPANSIN A6)	AT2G28950	-1.65	G1
259839_at	proton-dependent oligopeptide transport (POT) family protein	AT1G52190	-1.70	G1
245296_at	ATOPT3 (OLIGOPEPTIDE TRANSPORTER); oligopeptide transporter	AT4G16370	-1.71	S

249983_at	curculin-like (mannose-binding) lectin family protein	AT5G18470	-1.77	S
264348_at	NRT1.1 (nitrate transporter 1.1); transporter	AT1G12110	-1.89	S
246919_at	unknown protein	AT5G25460	-2.02	G1
265042_at	acid phosphatase class B family protein	AT1G04040	-2.22	G1
260924_at	protein kinase family protein	AT1G21590	-2.27	G1
255524_at	pectinesterase family protein	AT4G02330	-2.45	G1
256787_at	ATBFRUCT1/ATCWINV1	AT3G13790	-2.93	S

Table S4. Overlap between genes affected in *IOE-OBP1* plants and proliferation associated genes identified by Beemster *et al.* (2005).

AffyID	Description	Locus	Change (log_2)
252691_at	kinesin motor protein-related	AT3G44050	4.01
246415_at	Unknown protein	AT5G17160	3.83
248691_at	Unknown protein	AT5G48310	3.40
253978_at	Unknown protein	AT4G26660	3.38
246557_at	Unknown protein	AT5G15510	3.35
259978_at	CDKB2;1	AT1G76540	3.21
247039_at	ATEB1C	AT5G67270	3.21
261780_at	CYCB2;4	AT1G76310	3.11
261660_at	HIK (HINKEL); microtubule motor	AT1G18370	3.07
245343_at	binding	AT4G15830	3.02
248057_at	Unknown protein related	AT5G55520	2.95
259290_at	CYCB1;3	AT3G11520	2.83
255265_at	ATK5	AT4G05190	2.79
259815_at	Unknown protein	AT1G49870	2.71
258067_at	mitotic spindle checkpoint protein	AT3G25980	2.70
257740_at	zinc finger	AT3G27330	2.68
257290_at	Unknown protein	AT3G15560	2.66
251067_at	unknown protein	AT5G01910	2.66
254964_at	high mobility group (HMG1/2) protein	AT4G11080	2.60
255236_at	calcium-binding EF hand family protein	AT4G05520	2.60
254233_at	high mobility group (HMG1/2) protein	AT4G23800	2.55
248413_at	PLE (PLEIADE)	AT5G51600	2.52
256864_at	TOPII (TOPOISOMERASE II)	AT3G23890	2.51
257005_at	Unknown protein	AT3G14190	2.46
255507_at	ATIMPALPHA3/MOS6	AT4G02150	2.45
264802_at	SYP111 (syntaxin 111)	AT1G08560	2.44
257115_at	kinesin motor family protein	AT3G20150	2.39
255460_at	Unknown protein	AT4G02800	2.37
250228_at	WD-40 repeat family protein	AT5G13840	2.36
266655_at	ATAUR2	AT2G25880	2.31
252148_at	male sterility MS5, putative	AT3G51280	2.30
262109_at	ATCSLD5	AT1G02730	2.28
261859_at	UBC20	AT1G50490	2.25
252077_at	Unknown protein	AT3G51720	2.25
251527_at	Unknown protein	AT3G58650	2.21
253403_at	ATAUR1	AT4G32830	2.20
262081_at	ZCF125; microtubule motor	AT1G59540	2.19
249060_at	SNF7 family protein	AT5G44560	2.18
245739_at	CYCA1;1	AT1G44110	2.15
247134_at	Unknown protein	AT5G66230	2.13
252078_at	IMK2	AT3G51740	2.12
252089_at	similar to Os03g0174200	AT3G52110	2.06
258480_at	Unknown protein)	AT3G02640	2.05

263017_at	CYCB2;1	AT2G17620	2.04
258859_at	hydroxyproline-rich glycoprotein protein	AT3G02120	2.02
249644_at	Unknown protein	AT5G37010	1.99
258098_at	PAKRP1L; microtubule motor	AT3G23670	1.97
262752_at	CYCB3;1	AT1G16330	1.97
247603_at	chromosome-associated kinesin	AT5G60930	1.96
246505_at	Unknown protein	AT5G16250	1.94
246920_at	plastocyanin-like	AT5G25090	1.93
258867_at	Unknown protein	AT3G03130	1.93
250785_at	protein kinase-related	AT5G05510	1.91
249836_at	HMGB6 (High mobility group B 6)	AT5G23420	1.89
261364_at	dynamin family protein	AT1G53140	1.88
262758_at	F-box family protein	AT1G10780	1.87
267636_at	Unknown protein	AT2G42110	1.83
260910_at	importin alpha-2 subunit, putative	AT1G02690	1.83
253480_at	plastocyanin-like domain-containing	AT4G31840	1.81
253148_at	CYCB2;2 (CYCLIN B2;2)	AT4G35620	1.77
267232_at	Unknown protein	AT2G44190	1.74
259930_at	forkhead-associated domain-containing	AT1G34355	1.73
257134_at	Unknown protein	AT3G12870	1.72
265349_at	kinesin motor protein-related	AT2G22610	1.71
261384_at	similar to Os03g0747100	AT1G05440	1.68
253357_at	dem protein-related	AT4G33400	1.67
259851_at	kinesin motor protein-related	AT1G72250	1.64
266427_at	Binding	AT2G07170	1.57
257020_at	WD-40 repeat family protein	AT3G19590	1.56
262802_at	CDKB2;2	AT1G20930	1.56
254109_at	SKS1 (SKU5 SIMILAR 1)	AT4G25240	1.53
251822_at	unknown protein	AT3G55060	1.51
262558_at	similar to Os03g0780200	AT1G31335	1.49
266959_at	minichromosome maintenance protein	AT2G07690	1.39
255597_at	zinc finger (DHHC type) family protein	AT4G01730	1.36
251778_at	ATROPGEF6/ROPGEF6	AT3G55660	1.35
259686_at	scarecrow transcription factor	AT1G63100	1.30
264377_at	plastocyanin-like domain-containing	AT2G25060	1.30
251660_at	unknown protein	AT3G57160	1.19
261761_at	Unknown protein	AT1G15660	1.18
246899_at	Unknown protein	AT5G25590	1.13
252877_at	similar to Os08g0290400	AT4G39630	1.08
257714_at	histone H3	AT3G27360	1.07
249115_at	ZLL (ZWILLE)	AT5G43810	1.06
261058_at	HTR12 (Centromeric histone H3)	AT1G01370	1.05
247671_at	cytoplasmic linker protein-related	AT5G60210	-1.12
266790_at	ATEXPA6	AT2G28950	-1.65

Table S5. List of core cell cycle genes and genes encoding for cell wall loosening enzymes that expression was changed in *IOE-OBP1* plants 10h after DEX application.

AffyID	Description	Locus	Change (log_2)
259978_at	CDKB2;1	AT1G76540	3.21
262802_at	CDKB2;2	AT1G20930	1.56
260061_at	CDKD;1	AT1G73690	1.58
245739_at	CYCA1;1	AT1G44110	2.15

246938_at	CYCA2;1	AT5G25380	2.56
250369_at	CYCA2;2	AT5G11300	2.62
260329_at	CYCA2;4	AT1G80370	1.14
253051_at	CYCB1;1	AT4G37490	2.00
259290_at	CYCB1;3	AT3G11520	2.83
267618_at	CYCB1;4	AT2G26760	3.06
263017_at	CYCB2;1	AT2G17620	2.04
253148_at	CYCB2;2	AT4G35620	1.77
261780_at	CYCB2;4	AT1G76310	3.11
262752_at	CYCB3;1	AT1G16330	1.97
264697_at	CYCD1;1	AT1G70210	1.92
247034_at	CYCD3;2	AT5G67260	1.48
252189_at	CYCD3;3	AT3G50070	2.56
247190_at	CYCD4;1	AT5G65420	1.51
257483_at	KRP7	AT1G49620	1.67
264061_at	CKS2	AT2G27970	2.78
263955_at	E2Fa	AT2G36010	1.29
261741_at	E2Fc	AT1G47870	1.17
257524_at	DEL3	AT3G01330	2.20
262106_at	WEE1	AT1G02970	1.06
266873_at	CYCU4;1	AT2G44740	-1.23
256894_at	CYCU1;1	AT3G21870	-1.35
250992_at	ATEXPA9	AT5G02260	2.82
258877_at	ATEXPA13	AT3G03220	1.27
267158_at	ATEXPA3	AT2G37640	-1.49
266790_at	ATEXPA6	AT2G28950	-1.65
261266_at	ATEXPA10	AT1G26770	-1.32
261226_at	ATEXPA11	AT1G20190	-2.28
258003_at	ATEXPA5	AT3G29030	-2.06
256299_at	ATEXPA1	AT1G69530	-2.82
255822_at	ATEXPA8	AT2G40610	-3.63
252563_at	ATEXLA1	AT3G45970	-1.65
245463_at	ATEXLB1	AT4G17030	-2.40
247162_at	XTH6	AT5G65730	-2.49
252607_at	XTH31	AT3G44990	-2.22
247866_at	XTH25	AT5G57550	-1.90
261825_at	XTH8	AT1G11545	-1.87
266215_at	XTH4	AT2G06850	-1.58
253608_at	XTH19	AT4G30290	-1.19

Table S6. Ploidy measurments. a) Ploidy distribution in hypocotyls of dark or light grown one week old seedlings. Data are means ± STDEV (n=3); no STDEV (n=1). Each sample was pooled from approximately 30-50 seedlings growing on separate agar plates. b) Ploidy distribution measured in fully developed leaf six. Data are means ± STDEV (n=3-5). Bold font and shading indicate values that are significantly different from the wild-type plants (Student´s t-test; p< 0.05).

a)	Mock Control	DEX Control	Mock IOE#7	DEX IOE#7	Control	35S#1
Dark Grown Hypocotyls						
2c (%)	0.27±0.01	0.25±0.03	0.26±0.02	0.26±0.02	0.31±0.03	0.28±0.03
4c (%)	0.22±0.02	0.23±0.02	0.21±0.02	**0.33±0.03**	0.22±0.01	**0.27±0.02**
8c (%)	0.37±0.05	0.35±0.06	0.34±0.01	**0.31±0.05**	0.21±0.02	**0.31±0.01**
16c (%)	0.14±0.08	0.17±0.09	0.20±0.01	**0.10±0.01**	0.26±0.02	**0.14±0.01**
Light Grown Hypocotyls						
2c (%)	0.26	0.29	0.24	0.21	0.29	0.29
4c (%)	0.30	0.31	0.30	0.42	0.28	0.32
8c (%)	0.34	0.30	0.38	0.30	0.30	0.30
16c (%)	0.10	0.10	0.09	0.07	0.13	0.09

b)	Control	35S#1
Leaves		
2c (%)	13.66±1.53	15.64±2.84
4c (%)	23.15±3.03	**45.71±12.79**
8c (%)	42.38±7.18	33.77±14.06
16c (%)	18.96±8.69	**4.44±2.36**
32c (%)	1.86±0.87	**0.45±0.47**

Table S7. List of Q-RTPCR primers.

Locus	Gene name	Forward primer	Reverse primer
AT3G18780	Actin2	TCCCTCAGCACATTCCAGCAGAT	AACGATTCCTGGACCTGCCTCATC
AT4G05320	UBQ10	CACACTCCACTTGGTCTTGCGT	TGGTCTTTCCGGTGAGAGTCTTCA
AT1G13440	GAPDH	TTGGTGACAACAGGTCAAGCA	AAACTTGTCGCTCAATGCAATC
AT1G13320	PDF2	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGCTTGGT
AT2G32170	EXPRESSSED	ATCGAGCTAAGTTTGGAGGATGTAA	TCTCGATCACAAACCCAAAATG
AT1G44900	MCM2	ACGAGAATCAATGAACGGAC	CAGACATTCGGATCATGGAC
AT4G02150	SNC1	GTTCTCACAGATGCATGCTG	GACGAATGACCTAAGAGCTG
AT5G33300	KINESIN1	AAGTTCAGAGCGAACCAGTG	GGTTGAAATGGCTCCGAGTT
AT3G44050	KINESIN2	AGATTGCAAAAGCTTGTCGAC	ATCATCACCAACCATGCCTG
AT5G17160	EXPRESSED	AATGGTGGACCAAACCGACA	TTCAGAGATAGTGCCGTCTG
AT1G76540	CDKB2;1	CACGTCGTCAGGTTAATGGA	TGTTCTTGCCAGTGCTACGG
AT2G27970	CKS2	TCGGAAAGTGAATGGAGAGC	ATCGCGTAATGAACCCATCC
AT5G11300	CYCA2;2	ACCATCCTTGGAACCCTACT	GGTTGTATTTCTCACGGGTG
AT3G11520	CYCB1;3	TCTCAGCTCATGGATTGCTC	TCTTCAACACACCACGTAGC
AT1G76310	CYCB2;4	AAGCGCTTCTGGAATGTTCG	CCAGCTGGTTCAATTCTTGC
AT5G67260	CYCD3;2	CTCCAAGTGGAAGAAGCAAG	GGGTGCATTCTCCATTGAAG
AT3G50070	CYCD3;3	CTCTCCATTATTCCAGATTCG	GAGTAGAGTCATGAGCTGAG
AT5G65420	CYCD4;1	CCTAGTGGCAAAGGTTGGAT	GCTGCCAATGATAAACAAGC
AT3G01330	DEL3	GAACTCAAGGAAGAAGGGATG	AAGGTTCTTCTCTTTCACAACC
AT2G36010	E2Fa	CTGACTCTGGTATCGATTGG	CATTCCTGGTGGTGGAGTAC
AT2G07690	MCM5	ATAGCAACCCTATCTTCCGG	GGTGCTATGGTTTTGGCTGA
AT1G49620	KRP7	GTTTCGCTGCTGGAACAACC	TTCTGCTCCGTCGCGTTATC
AT5G67480	TAZ	GCAAATACGCGGAAAGAGAG	AAATCTTTATCGTGTGGCCC
AT2G40970	MYB	GCCGATCAGTAGCCGAATCAGAGA	TTAGGAGAATGCAAGGCGGGAACG
AT3G50410	OBP1	AGCACTTCCCTTTCCGTCGCTTTC	GTTCCGTTACAAACGACGCCTGTT
AT2G34140	DOF	CACTCCTCAACGAGCAAGCCATTT	TGTCGTGGTTGGTATGCTTGGAGA
AT2G02820	MYB88	TGTAGTAGCCCACAATGCCACAAG	TCCTCACCGTCACTCTCTTTCACA
AT2G47730	GST6	ACGAGAAAGATCTCCAGTTCG	CTCTTGACTCGAAAAGCGTC

Table S8. Q-RTPCR primers used for ChIP

Gene	Region (relative to 5'UTR)	Forward primer	Reverse primer
UBQ10		CACACTCCACTTGGTCTTGCGT	TGGTCTTTCCGGTGAGAGTCTTCA
AtDOF2;3	75-206	GCAGGAATTGGAGAAGAAAAG	ACGTGTAGATGTGAATTCAAG
AtDOF2;3	600-695	TCTCTCCGTTTCACAATATAAG	TACAGTCTCTTTTATGATTGGC
AtDOF2;3	674-826	AGTTCAGCCAATCATAAAAGAG	TCGTTTTGGATATTTCCTCCG
AtDOF2;3	807-932	CGGAGGAAATATCCAAAACG	AACTCGTGACTCGTGCACTG
CYCD3;3	8-157	AATGCCAAAGAAAGTGAAGGG	CGCCAAACATCCGAATATTAA
CYCD3;3	328-380	AAGCTTATACTCACGTGCTTA	AGGGATCAAAAGCGTGTAAC
CYCD3;3	495-637	ACCTTTCGGTTTCAAGCATC	ACAGTGACTCTACACATTACG
GST6		CGTTTCTAGGAGAGGTTGAC	ACTGTTCTAAGGTAATCCTTCC

Table S9. Q-RTPCR primer platform for core cell cycle genes and TFs that expression oscillates during cell cycle (Menges *et al.,* 2003). The majority of the TF primer sequences were taken from Czechowski *et al.* (2003)

Locus	Gene	Reverse	Forward
Transcription factors			
AT1G01530	MADS	CTGAAAGCCGCACAAAGCTC	CAGCCATCACCTCAGTTAAAGACTC
AT1G01720	NAC	TCAGGCTGGATGATTGGGTTCTCT	GCCTCTCGGTAGCTCCTTTTTTGT
AT1G02250	NAC	AGTGCATCCACATCAAGAAAGCCA	GAGGGATCGTTGTTTCACAGCCAA
AT1G04020	putative DNA-binding	CCCTTGCCAGTTCTGCTTTC	AATGGGCACCGCAAGTTTAC
AT1G04250	Aux/IAA family	GCCAAGGCACAAGTTGTGGGAT	TTTGGCAGGAAACCATCACGTTCT
AT1G04550	Aux/IAA family	ACCACTTGACTTGAACGAGGAGGA	TGGGTCTAAACGCTCTGCTGAATC
AT1G08810	MYB	TGGACGCCCATTTGTTACCCAA	ACACTGGGTTATTGAGATGCAGCA
AT1G17680	General Transcription	TTGATTAACGCAGCTCCCAC	ACAAGCCGACATCAAGATGC
AT1G19050	ARR-A	AAGTGACGACTGTAGAGAGTGGAA	TCCTGAAAGTCCTGGCATTGAGTA
AT1G21910	AP2/EREBP	GCTGCTCAAGCTGCCAACTCATTT	TGACGAGACGGCTGATGAAGTAGG
AT1G22070	bZIP	GAGTTTTGTGAACCAGGCGGATCA	GAGCAGCCTGTCTTGTCGTCAATA
AT1G26760	SET	TAACGAAGGAGTTGGGTGTC	CTCTCCTCTCATTGCTGTTC
AT1G277300	C2H2 zinc finger	TCGAGCACTGGACAAAGGGTAAGC	CCTCAGTGAGGTTTTGGTGGTGGA
AT1G284600	MADS	CATCATCTTCTTCGCCTTTTCG	ATGTGTGGTGGAAAGTGGATCC
AT1G299500	bHLH	CCAACGCCAAGTTTCTGAG	AGCTTGCGTTCTTGATGAAG
AT1G304900	HB	TGCAGCAGGGATATGCGAATCTTC	ACCGTCGCTTGCTCATACGAAAC
AT1G313200	AS2	GCTCATCGGCCGGAAAATAC	GAGCAGCATGGTTTACGAGG
AT1G504200	GRAS	CCTGCAACATCGCATAATAG	CCTGCGAGGGATTTGAGAGA
AT1G631000	GRAS	TGCTGCTACTGCTTCACTCCTCAA	TTGGCAGGTTGTTCGAGTTCATCA
AT1G634800	putative DNA-binding	ATCCGCCTGTTCGACTTCTG	ATACGAGGGACCTTTTGAGAT
AT1G648600	General Transcription	TCGAATGATCTCTTTTTCCCG	GGCACTCAAGGAGGAAGTAAG
AT1G685500	AP2/EREBP	CCTTGTTTCCACCAGCAGGAACAT	CTGCATCAGCTCTCACTTGTGTCA
AT1G696900	TCP	GCAGCATTCAACGCCGCTAAAACT	TTTCTGGACAGCCTTTGGCTTCTG
AT1G774500	NAC	CCGACATGGCTTTGTACGGTGAAA	AGCTGCACGGTTGGGTCTTGAA
AT2G028200	MYB	TCCTCACCGTCACTCTCTTTCACA	TGTAGTAGCCCACAATGCCACAAG
AT2G24260	bHLH	GCCACTTGATGTTCCGTCAT	TTCTCAAATCTCCGAGGCTG
AT2G256400	transcription regulator	TCGCCACTTCTTAGAATGCCGATG	CGTTTATGGGAAGGTGTGCTCCAG
AT2G261500	HSF	TGCTTTTGTCCTGCTAAAAACCCT	TCTCAACAACTCTCCTTCCACGTT
AT2G272300	bHLH	AGTTTCGATGACTCCCTTCA	CCAAGTCGAGATGTTATGCG
AT2G305900	WRKY	GCCCTTGATGGGCTTCTGACCATA	GCCACTGCGCTAAGAAGAGGAAAC
AT2G311800	MYB	CGCAGGTTTACTTAGATGTGGGAA	TTGCCTAAGCTTTCATGCAGATTG
AT2G333100	Aux/IAA family	TAAACCGGCTGCTTTCGCTGTCTC	GCTAATGGACTCGCTGCACGAAAT
AT2G341400	DOF	TGTCGTGGTTGGTATGCTTGGAGA	CACTCCTCAACGAGCAAGCCATTT
AT2G407500	WRKY	TGCTACACTAGAAAGACGAGATCA	TTCCTCCAAGCATATCTGTCTTCA
AT2G409700	G2-like	TTAGGAGAATGCAAGGCGGGAACG	GCCGATCAGTAGCCGAATCAGAGA
AT2G423000	bHLH	GAACGCGAGAAGAAGGTCAAAAGC	TGGCAACTTGTCTGACTCCACG
AT2G431400	bHLH	TCTACTTGGTGCTGAAGACC	AAAGGGAGAGAAGAACGAGG
AT2G475200	AP2/EREBP	TGGCCTCTGCCTTATCCCTCTGTA	GCGTAAACCCGTCTCAGTGAGTGA
AT3G067400	C2C2(Zn) GATA	TGGTACCAGCAAAACCCCTCT	GCATGCGTTACAAAGCGACTT
AT3G073400	bHLH	GCCTGGTGTTCACTGACGATAA	GTCCCTGGATGCAACAAGGTT
AT3G100400	Trihelix	TCTCCGCAATTCTCGCCATTTTCC	TTCCGATTCCGAGTCAGCAGCAGA
AT3G130400	G2-like	CTTCCATCTGCATACGCAGTGCTT	CTGACGAGAAGAAGAAAGGGGCCA
AT3G147400	C2H2 zinc finger	CTTTTCTCGGTTCTTCGACG	GCTTCTTGCTATGGGAATCC
AT3G162800	AP2/EREBP	CCGGAGCCTCATCCTTCTCTAACA	GTCATCATCGCCGCATGATATCCA
AT3G182900	C2H2 zinc finger	TAACAGTGCATCAAGCATACC	TGCTTCCAGGCTTACACTTG
AT3G230300	Aux/IAA family	GCTCGGGGTAGTTTTTGTATGTCT	ACCTCCTACCAAAACTCAAATCGT

AT3G232500	AP2/EREBP	AAAGACCAGCTTGCTTAGGGAGGG	TCCATGCTGTGAGAAGATGGGGT
AT3G245000	transcription regulator	ATCATCAACCTCACCTCTGC	CACGAAGAAGCTGGAAGAAG
AT3G27010	TCP	TGGCCATAACCCAGTTGGTA	GATGGTGGGTCTAGTAGTAG
AT3G284700	MYB	TCACCGGGTCTATCCCCATTTTCA	ATTGGCAGCAGGTGGTCTTCCA
AT3G289100	MYB	TGCTGCATCTAAGCAGCCCAGTAT	AGGAGGAGTGAAGAAAGGGCCAT
AT3G467700	B3	GAGTCATATCAACCGGAAAC	TCGCCAAGAAGAGGTGGTAC
AT3G500600	MYB	ATTCCGTCACCGTCGTCTCCTGTT	GTAAAACCGCCGGAAATCGGCA
AT3G504100	DOF	GTTCCGTTACAAACGACGCCTGTT	AGCACTTCCCTTTCCGTCGCTTTC
AT3G521700	putative DNA-binding	TTCAGAACTGCCAAGAGAGG	AACCTGAATCGTGGAAAGGG
AT3G543200	AP2/EREBP	GCGGCGGAGAGAAGCCAAATATTC	TTCCGGCAGAGACGTACACAAAGG
AT3G544300	C2H2 zinc finger	CTCGTTGTTGCTTATCGCTG	ACTTCCGACAACCACTTCTC
AT3G559800	C3H zinc finger	TTGAGCTGCAAAGCCGGTGGAGTA	TCCTCTCCAAGAAACGGCGGATCA
AT3G564000	WRKY	ACACGTCTCCGATCTCTTTTTTCT	GTTTGAAGATTCCGGCGATAGTC
AT3G613100	putative DNA-binding	TAGACTTCCAGTGCGGTTTG	ATACGAGGGTCTATTCGAGC
AT3G621000	Aux/IAA family	TCTCCAACCATCATCCAGTCACCT	TGCTTCAATCCTTTGGGCTGAAGA
AT4G012500	WRKY	TCTATTTCGCTCCACTTGTTTACG	AAAGGTTCACCATATCCAAGAGGA
AT4G110800	HMG	TCCAGCCATCTTAGCGACCTCAAT	GCAAACGAGAGGAGAGCTGCTTTA
AT4G215500	B3	TTGCGCCTCTGCGTATGAACAAGG	GCTGCACGAAATGAACACCTCCAT
AT4G238100	WRKY	GCCTCTCTCTGGGCTTATTCTCAC	TTTGCCGATGGAGGAGGTTCTAGC
AT4G255300	HB	TGTGCCACATTCTCCACCTG	GTCCAGGATTGTCTGCAAAAG
AT4G308600	SET	CGGAATGGTCTGTTTCCAC	GCAGGGTTCAATGCATAAGC
AT4G328800	HB	TGGAAGACGGGAGCCTTGTGATAT	CGAAATGAGGAGACGGAGGCATAC
AT4G369900	HSF	TCGTCAGCTCAACACTTACGGATT	CCGTCGTATGTCCGTCAACAGAT
AT5G071000	WRKY	GGAGACGGCAAAATGTTGGAGGAA	TTCACTATGCCTCCTGGCCTTACT
AT5G075800	AP2/EREBP	GCTTCATATTTCCCGGCGTCAAGA	TGCTGCAAGAGCCTATGACTGTG
AT5G081300	bHLH	TGGGAAGAAGGGACAGACAAGGAA	AGTTGCTGCAACATCAGCTCTCAT
AT5G105100	AP2/EREBP	ATTGCCTCCCTCAGGAGGAAGAGT	CCTTGCTTGCCAACGTCCTTGTT
AT5G115100	MYB	CTGATCCAAAAGGCCAGACGACAT	TGCCTGGAAGGTCGGATAATGGAA
AT5G123300	C2H2 zinc finger	ATTAGGCGGGTCGAGAATAG	TGCATTTAGGTGGTTCAGCC
AT5G185500	C2H2 zinc finger	CTTCTGTTCTGAGACCTCCC	ACCCGATGAACCCGATTGTA
AT5G225700	WRKY	GGGGAGCCCCTCCAAGAAAAGAAA	TCCGGTGAATCGTCCCTCCAATT
AT5G251900	AP2/EREBP	TCAAGAGAGGGTGACGAATTTCGG	ATGGCACGACCACAACAACG
AT5G439900	SET	TATCTCTCCAGCAAGCTCAC	GCATTCACAACAAGCTGCAG
AT5G465900	NAC	TTCTCCCGTTGGTATCCAGAGGTT	GTCCCTTGAGCAGAAGAAGTACCA
AT5G511900	AP2/EREBP	TCAAGTACATCTGGAAGTCGAAGC	AGAGGGAAGAAGCTCAGATCACTA
AT5G562000	C2H2 zinc finger	CAACAGGTCAAGCTCTTGGTGGT	CCACAGTAGAAACTGGTGCAGTCC
AT5G594500	GRAS	GAACCGTGTCTCTTGGAGAG	AACGGTCGTCAACTGCATTC
AT5G604400	MADS	CGAGTTGAGATAACGCAAGTTCC	TCATCTTACTCAGGTGTTGAGTCA
AT5G616000	AP2/EREBP	TCCCCTGTAGTGCCTCTCTTCTTC	TAACCAGCGTAAACCGCCCTTACC
AT5G618900	AP2/EREBP	CCGCAGATTCAGCGGTTTCAAAT	AAGACCAAGGGGACTTGAGGAGGA
AT5G637000	putative DNA-binding	AACACCACTTGCCATACCAG	AGATCTTGCAGGTTACAGGC
AT5G671800	AP2/EREBP	TAATCGCCGCTCGATCATAGGCTC	TGGGACTGTGGGAAGCAAGTGTA
AT5G674200	AS2	ATTCCGATTGCTCCGTTAAC	AACGTCCTGCTTTGTTTCAG
AT5G674800	TAZ	AAATCTTTATCGTGTGGCCC	GCAAATACGCGGAAAGAGAG
Core cell cycle genes			
AT5G03455	CDC25	CTTGTCATCAAACGAGCCAC	AATCATGGGAACTCCGTACG
AT3G48750	CDKA1	CTAGGTCCGTTGGTTTCCAT	TGGATGCATCTTTGCCGAGA
AT2G38620	CDKB1;2	GGGCTAAAGTCCTAAACTGG	TGAGGCTTAAGATCCCTGTG
AT1G76540	CDKB2;1	CACGTCGTCAGGTTAATGGA	TGTTCTTGCCAGTGCTACGG
AT1G20930	CDKB2;2	ATCAGAAGCTTTCGTCAAGC	TGCCGATATTCTCTGTGCTG
AT5G10270	CDKC1	AGAAAAAGCGGCAACAGCAG	CTACAGACACTTTGATCGCC
AT5G64960	CDKC2	TCTTTGGATCACATGGCAGC	GCAGACCATTTCTTCAGGGA
AT1G73690	CDKD1	AGATCATGTCAGGCCACTGA	CTTCCGGGTTCTACTGAGAT
AT1G66750	CDKD2	ATGGTGGAGCGGGTGTATAG	CATTCCACCATTCACCAAGTA
AT1G18040	CDKD3	GATCAAAGAGGCACACTCCT	ATCCTCTGCATGTTATGTGC
AT5G63610	CDKE1	GAATTACCCAACTCGTCCAG	GTCAGGCTAGGATACTCATG
AT4G28980	CDKF1	CGCTGGTTTCTCCATCTTTG	CGCTGGTTTCTCCATCTTTG
AT5G63370	CDKG1	AAATGATGAGAACCCAGGCC	CGGGTAAGAGTGAGCTTGAC
AT1G67580	CDKG2	CCGAGCCAAACCAAAGTCAC	AGAGGGCGTCAAGTATCTTC
AT1G57700	CKL10	GGTACAGCTTTGGACTCTCT	GGAAGCAAGACGACGGAAAG
AT1G09600	CKL11	AACCGCTAACTGAATCTGCC	AGAGAGAATGCACTGCTCAG
AT4G10010	CKL13	ACCAGGAGGCATCAATGGTC	CCGTACAAACCACAACGACA
AT1G53050	CKL15	GCTCTTTGCTGGGAGGATAT	AAGAGTGAGTTCTTCTCCAC
AT5G39420	CKL1	TGTGTTGTTGTAGTGGCTCG	AAGAAAGGACACGCTTGAGG
AT1G74330	CKL2	GCCACCTTTAATATCCCTTC	ACGACGCCACAGATTAAGTG
AT1G18670	CKL3	CTTGCAGCATACATCTCGTG	TCTGTGGAGTAGTGAAGTCG
AT4G22940	CKL4	AAAGCCACGGTCTTGTTACG	GCAAGAACAAATTGGGGGAG
AT5G44290	CKL5	ATCGGACCTGAGTGAGAGAC	GAAGCTGTGTTCCGGGTAAT
AT1G03740	CKL6	GCCTATAAGGAATCGCGGTT	AACCGAGGTAGAACAATTGC
AT3G05050	CKL8	CTGTGGTAAAGGCTTGTGATG	GTAACGAGGTCGAGCAGTTG
AT1G54610	CKL9	TGCCGATTCTTCCTTGCACC	TGAAGAAACTCGGAGACAAAG
AT2G27960	CKS1	TTTCGAATACAGGCACGTCG	ACTCCTATCGCTCGCCATTC
AT2G27970	CKS2	TCGGAAAGTGAATGGAGAGC	ATCGCGTAATGAACCCATCC
AT5G50860	CKL7	AAAACTTCTGATCTGCTCCTG	GTGGGGCTTCTTCTAGAAAG
AT1G71530	CKL12	GCTCTGTACCATAAGGTCAC	TTGGTGATTTCGGATTGGCG
AT1G33770	CKL14	TGGATTCGTCTCTCGTGCTC	CACCCGCAACGACATACATG
AT1G44110	CYCA1;1	CTTCGTGCTTCTGAGGCTAA	CTTCAGACACCTCAATAAGCC

AT1G77390	CYCA1;2	TACGCTATGCTTCGATACGC	TCTAGCGTAGCATTCCATGG
AT5G25380	CYCA2;1	CCGCGAAATAGTTGGCTAAG	CGTTTCTCAGGCGGTTCATT
AT5G11300	CYCA2:2	ACCATCCTTGGAACCCTACT	GGTTGTATTTCTCACGGGTG
AT1G15570	CYCA2;3	CCTTTGGTGTTAAGCTGCAG	AACCACCCATGGAATCCAAC
AT1G80370	CYCA2;4	GACAGATGCTTTGAGATCGG	ATCAAGCCACCCTTGGAATC
AT5G43080	CYCA3;1	CATTCTTTCAAATCACCCGC	CTAGGTTCATCATCCGTCCA
AT1G47210	CYCA3;2	CTCAAGTTGACGAAGATACTC	AGCTCCGGTGGCTATTATTG
AT1G47220	CYCA3;3	AGCAACAAAGAGGCTCTAGC	TCTAAGACGGTTCATAAGGG
AT1G47230	CYCA3;4	CCGTGTAAACCGTCTTAGG	AGAAGTGGTGTCGATGGAGG
AT4G37490	CYCB1;1	CCTCGCAGCTGTGGAATATG	CCTCCATTCACTCTCAACAG
AT3G11520	CYCB1:3	TCTCAGCTCATGGATTGCTC	TCTTCAACACACCACGTAGC
AT2G26760	CYCB1;4	CTTACTCTCTGAAGCCGAGT	ATGAGATTATGGAGCATGCG
AT1G34460	CYCB1;5	ACATACTCCACTGCAGCCAC	CGAGACGAAGAGGAATGCTC
AT2G17620	CYCB2;1	CAAACTTGTCATGTACCTCG	CGAGAAGATGAGAGCAATAC
AT4G35620	CYCB2;2	TCGATGAGCCAGTCAATAAG	CGAGAGATTTAGCTGTGTTC
AT1G76310	CYCB2:4	AAGCGCTTCTGGAATGTTCG	CCAGCTGGTTCAATTCTTGC
AT1G20590	CYCB2;5	GGCCTCAGCAATCTACACTG	CATGCCAGTAGCTGTTCTTC
AT1G16330	CYCB3;1	CTGCTATGTGCATCAGCCAT	AACAGTCCTTCATCTGGGAG
AT5G48640	CYCC1;1	ATGAACCAGATTACATGGGG	TATGCATGCTAGCGCTATGC
AT5G48630	CYCC1;2	CCATATCGATCTCTGCCTGA	CAAGACCCCAAGTTAGATGG
AT1G70210	CYCD1;1	CTTCACTCCTGCAACCTGAA	AAACGAGTGGTTGGCCAATG
AT2G22490	CYCD2;1	ACCATTTTGGACATCTGTGC	GTCTTTCGGCAATTCATAGG
AT4G34160	CYCD3;1	AAGGACACCGAGGAGATTAG	CCTCTCTGTAATCTCCGATTC
AT5G67260	CYCD3:2	CTCCAAGTGGAAGAAGCAAG	GGGTGCATTCTCCATTGAAG
AT3G50070	CYCD3:3	CTCTCCATTATTCCAGATTCG	GAGTAGAGTCATGAGCTGAG
AT5G65420	CYCD4:1	CCTAGTGGCAAAGGTTGGAT	GCTGCCAATGATAAACAAGC
AT5G10440	CYCD4;2	CCTCAAGAGACTGAGAAACG	CCACTAGGTAAGTCATGAACAG
AT4G37630	CYCD5;1	CTACGCATTCTCTTGGCTGG	GACACCTCCACCGGAAATAG
AT4G03270	CYCD6;1	TGTCTAGCGAAGATATGCCG	CACCGGAAGATCAGAAACAG
AT5G27620	CYCH1	GAAAGAGGAGTGGAGCATCT	AGAGAGTTTGCTCAAAGGGG
AT2G26430	CYCL1	AGGGAGTGGTACTTGAAACC	TTGGCCAATGATAGTCTGCG
AT1G27630	CYCT1	AAGTCGCTATTGTCTGCCAG	GAAGCTTCATGTGTCCCAGG
AT4G34090	CYL1	ACTTGGATTGGAGCTTTGCC	TGTCCGTATGAGGAGGTTGC
AT5G14960	DEL2	GGGCTTGTTGCAAGAAGTGGGAA	AGCACGAGGAACAGCTCCAAAACC
AT3G01330	DEL3	GAACTCAAGGAAGAAGGGATG	AAGGTTCTTCTCTTTCACAACC
AT5G02470	DPa	TTACAAGGAGGTTGCAGACG	ATTGAGCGCATCGTAGACTC
AT5G03410	DPb	ACACCAGAAGCTCCTTGAGAACCA	GCGACACTACTTTTCAACGCCTGA
AT2G36010	E2Fa	CTGACTCTGGTATCGATTGG	CATTCCTGGTGGTGGAGTAC
AT5G22220	E2Fb	CACGTCTATTGGTCCCATTGTGCT	GAGGTTCCAGATCCTGATGAGGCT
AT1G47870	E2Fc	GAAGGGTGCTGACAATCTTGGACA	TCCAACCTGCTTTCCTCAGATTGC
AT2G23430	KRP1	GTATCGACGGGGTACGAAG	CGCCGATTCAAATTCCGATG
AT3G50630	KRP2	AGCTTCCTTCACCGTCTCAT	AGTGAGGAATCGATGAACATG
AT5G48820	KRP3	CAATTTCGTGAGTGTCCAAG	CTCTCCCTTGTGCTGTGTCT
AT2G32710	KRP4	AAGCTTTGTAGACGATCCCG	AGCTTCAACAGGACCACAAG
AT3G24810	KRP5	TTGTTGCTGTTCTGCGCTAG	ACAGGAGCATGATAAGTGATTC
AT1G49620	KRP7	TTCTGCTCCGTCGCGTTATC	GTTTCGCTGCTGGAACAACC
AT3G12280	Rb	CACATGCTTACCAGAGCCCT	TGCTGCTGCTGTTGTTCAAG
AT1G14750	SDS	CACTTCATGCCGGCTATATC	AACCTTACAACAGCATCCGG
AT1G02970	WEE1	GCACCAATTTGCCGGATTTC	CCTTGCCTCAAGAATCCGTA

Deutsche Zusammenfassung

Biologische Prozesse, wie beispielsweise das Wachstum von Organen und ganzen. Organismen oder die Reaktion von Lebewesen auf ungünstige Umweltbedingungen, unterliegen zahlreichen Regulationsmechanismen. Besonders wichtige Regulatoren sind die sogenannten Transkriptionsfaktoren. Dabei handelt es sich um Proteine, die die Aktivität von Erbeinheiten, den Genen, beeinflussen. In Pflanzen gibt es etwa 2000 solcher Regulatoren. Da sie wichtige Kontrollelemente darstellen, sind sie von großem wissenschaftlichen und biotechnologischen Interesse.

Im Rahmen der Doktorarbeit sollte die Funktion von drei Transkriptionsfaktoren, genannt OBP1, OBP2 und AtDOF4;2, untersucht werden. Sie wurden bei der Suche nach neuen Wachstumsregulatoren identifiziert. Als Untersuchungsobjekt diente die in der Öffentlichkeit kaum bekannte Pflanze Ackerschmalwand, lateinisch als *Arabidopsis thaliana* bezeichnet.

Um die Funktion der Regulatoren zu entschlüsseln, wurden an der Modellpflanze genetische Veränderungen durchgeführt und die Pflanzen dann mit molekularbiologischen und physiologischen Methoden analysiert. Es zeigte sich, dass OBP1 an der Regulation der Zellteilung beteiligt ist. Alle Lebewesen sind aus Zellen aufgebaut. Gelingt es, die Zellteilung gezielt zu steuern, kann damit beispielsweise die Produktion von pflanzlicher Biomasse verbessert werden. Das OBP1-Protein übt auch einen Einfluss auf die Zellstreckung aus und beeinflusst auch auf diesem Wege das pflanzliche Wachstum.

Die beiden anderen Proteine steuern Prozesse, die im Zusammenhang mit der Bildung von Pflanzeninhaltsstoffen stehen. OBP2 ist Teil eines zellulären Netzwerkes, dass die Synthese von sogenannten Glucosinolaten steuert. Glucosinolate kommen unter anderem in Broccoli und Kohl vor. Sie fungieren als Abwehrstoffe gegen Fraßinsekten. Einigen Glucosinolaten wird auch gesundheitsfördernde Wirkung zugesprochen. Das Protein AtDOF4;2 ist Komponente eines anderen Netzwerkes, dass die Bildung von Phenylpropanoiden steuert. Diese Substanzen haben strukturelle Funktion und spielen darüber hinaus eine Rolle bei der pflanzlichen Toleranz gegenüber tiefen Temperaturen.

Mit der Doktorarbeit konnte das Wissen über die Transkriptionsfaktoren erheblich erweitert und die Grundlage für interessante zukünftige Arbeiten gelegt werden. Von großer Bedeutung wird es dabei sein, die Netzwerke, in die die Transkriptionsfaktoren eingebunden sind, noch besser zu verstehen. Dann wird es möglich sein, auch Teilnetzwerke gezielt zu beeinflussen, was für biotechnologische Anwendungen, beispielsweise bei der Präzisionszüchtung von nachwachsenden Rohstoffen, von zentraler Bedeutung ist.

Curriculum Vitae

Personal details

Name, Last Name: Aleksandra Skirycz
Date of birth: 11.07.1980
Place of birth: Oława, Poland
Nationality: Polish

Academic qualifications

- **09.1995 – 06.1999** Secondary School number VII in Wrocław, Poland. Graduated with A-level exams from literature and Polish language (note: Very Good from oral exam and Good from written exam), biology (note: Very Good), English (note: Very Good).
- **10.1999 – 06.2002** Bachelor degree in Biotechnology, Institute for Biochemistry and Molecular Biology, Department of Natural Science, University of Wrocław, Poland. Final note: Very good. Work title: "Transgenic plants as a tool to elucidate carotenoids biosynthesis pathway in plants". Supervisor: Prof. Jan Szopa.
- **10.2002 – 06.2004** Master degree in Molecular Biology, Institute for Biochemistry and Molecular Biology, Department of Natural Science, University of Wrocław, Poland. Final note: Very good. Work title: "Expression of human dopamine receptor in potato (*Solanum tuberosum*) results in altered tuber carbon metabolism. Transcription factor Dof15 as a candidate gene regulating auxin/indole glucosinolate homeostasis in *Arabidopsis thaliana*". Supervisor: Prof. Jan Szopa, Dr. Isabell Witt and Prof. Bernd Mueller-Roeber.
- **09.2004 – 12.2007** PhD Degree in Plant Molecular Physiology, Max Planck Institute for Molecular Plant Physiology, Golm, Germany and University of Potsdam, Golm, Germany. Work title: "Functional analysis of selected DOF transcription factors in model plant *Arabidopsis thaliana*". Supervisor: Prof. Bernd Mueller-Roeber.

Scholarships

- **2001 – 2004** Scientific Scholarship granted by Wrocław University for the exam results.
- **02.2003 – 12.2003** Socrates/Erasmus Scholarship for gaining international experience. Max Planck Institute for Molecular Plant Physiology, Golm, Germany. **01.2005 – 01.2007** Schering Fellowship (awarded to a total of approximately 7 doctoral candidates per annum in area of biology, chemistry and medicine).

- **09.2004 - ongoing** Participation in the International PhD Program Integrative Plant Science.

Conferences

- **11–14.07 2004** 15th International Conference on Arabidopsis Research, Berlin, Germany. Poster: “Transcription factor Dof15 as a candidate gene regulating auxin/indole glucosinolate homeostasis in Arabidopsis.”
- **23-27.08 2004** 14th FESPB Congress, Kraków, Poland. Oral presentation during “Secondary Metabolism” session: “Transcription factor Dof15 as a candidate gene regulating auxin/indole glucosinolate homeostasis in Arabidopsis”.
- **15-19.06. 2005** 16th International Conference on Arabidopsis Research, Madison, USA. Poster: ”Transcription factor Dof15 as a candidate gene regulating glucosinolate metabolism in Arabidopsis”.
- **18-23.08 2006** 8th International Congress of Plant Molecular Biology, Sydney, Australia. Posters: “Dof19 – tapetum, shoot branching and flavonoid metabolism” and “DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in *Arabidopsis thaliana*”.
- **10-14.09 2006** The First International Conference on Glucosinolates, Jena, Germany. Poster: “DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in *Arabidopsis thaliana*”.
- **8.12. 2006** XVII. Berliner Botanischen Graduierten Kolloquium „Havel- Spree-Kolloquium“ Oral presentation: “Characterization of a transcription factor linked to cell cycle regulation in Arabidopsis”.
- **2 – 4.05 2007** EMBO Conference, Ghent, Belgium Poster: “Characterisation of a transcription factor linked to cell cycle regulation in Arabidopsis”.

Workshops and symposia

- **18 - 20.07.2005** Workshop on Molecular Interactions, Berlin, Germany.
- **17 - 21.10 2005** Career Training for European Researches.
- **26 - 27.01 2006** 15th New Phytologist Symposium: Networks in Plant Biology, London, UK.
- **3 - 5.04 2006** Workshop on Molecular Interactions, Berlin, Germany.

Publication list

- **Skirycz, A., Jozefczuk, S., Stobiecki, M., Muth, D., Zanor, M.I., Witt, I., Mueller-Roeber, B.** (2007) Transcription factor AtDOF4;2 affects phenylpropanoid metabolism in *Arabidopsis thaliana*. *New Phytologist* **175**, 425-438.
- **Stobiecki, M., Skirycz, A., Kerhoas, L., Kachlicki, P., Mueller-Roeber, B.** (2006) Profiling of phenolic glycosidic conjugates in leaves of *Arabidopsis thaliana* using LC/MS. *Metabolomics* **4**, 197-219.
- **Skirycz, A., Reichelt, M., Burow, M., Birkemeyer, C., Rolcik, J., Kopka, J., Zanor, M.I., Gershenzon, J., Strnad, M., Szopa, J., Mueller-Roeber, B., Witt, I.** (2006) DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in Arabidopsis. *Plant Journal* **47**, 10-24.
- **Mueller-Roeber, B., Witt, I., Zanor, M.I., Skirycz, A., Ruzcic, S., Riaño-Pachón, D.M., Caldana, C**. (2005) Pflanzliche Regulatorproteine. *BIOforum* **6**, 1-4
- **Gómez-Merino, F.C., Arana-Ceballos, F.A., Trejo-Téllez, L.I., Skirycz, A., Brearley, C., Dörmann, P., Mueller-Roeber, B.** (2005) Arabidopsis AtDGK7, the smallest member of plant diacylglycerol kinases, displays unique biochemical features and saturates at low substrate concentration. *Journal of Biological Chemistry* **280**, 34888-34899
- **Skirycz, A., Świędrych, A., Szopa, J.** (2005) Expression of human dopamine receptor in potato (*Solanum tuberosum*) results in altered tuber carbon metabolism. *BMC Plant Biology* **9**
- **Świędrych, A., Skirycz, A., Stachowiak, J., Lorenz, K., Szopa, J.** (2004) Catecholamine biosynthesis route in potato is stress effected. *Plant Physiology and Biochemistry* **42**, 593 - 600