Max-Planck-Institute of Molecular Plant Physiology Golm

Identification and analysis of new phloem proteins from *Brassicaceae* and *Cucurbitaceae*

Dissertation

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This Ph.D. Thesis is the account of work done between May 2002 and June 2005 in the department of Prof. Dr. Lothar Willmitzer in the Max-Planck-Institut für Molekulare Pflanzenphysiologie, Golm, Germany. It is result of my own work and has not been submitted for any degree or Ph.D. at any other university.

Die Dissertation ist das Ergebnis praktischer Arbeit, welche von Mai 2002 bis Juni 2005 durchgeführt wurde im Department von Prof. Dr. Lothar Willmitzer im Max-Planck-Insitut für Molekulare Pflanzenphysiologie, Golm. Ich erkläre, dass ich die vorliegende Arbeit selbständig und ohne unerlaubte Hilfe angefertigt habe. Es wurden keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, und die den benutzten Quellen wörtlichen und inhaltlichen Stellen sind als solche kenntlich gemacht.

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Abbreviations

℃	Celsius grades
µ	micro
1 DE	one-dimensional electrophoresis
2 DE	two-dimensional electrophoresis
aa	amino acid
AnnAt	*Arabidopsis thaliana* annexin protein
APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumine
cDNA	complementary DNA
dCTP	deoxy cytosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double strand DNA
DTT	dithiotreitol
EDTA	ethylenediamine tetraacetic acid
ESI	electrospray ionisation
Fig.	Figure
FW	fresh weight
g	gram
GC/MS	gas chromatography mass spectrometry
GRP	glycine-rich RNA-binding protein
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethansulfonic acid
IEF	isoelectrofocusing
IgG, IgM	immunoglobulin G, immunoglobulin M
kDa	kilodalton
KO	knock out
l	litre
m	mili
M	molar concentration (mol/litre)
m/z	mass to charge ratio
min	minutes
mRNA	messenger ribonucleic acid
MS	mass spectrometry

MSTFA	N-methyl-N-(trimetylsilyl)trifluoroacetamide
n	nano
NADP	nicotinamide adenine dinucleotide phosphate
NaOAc	sodium acetate
NCBI	National Center of Biotechnology Information
nd	not detected
OD	optical density
oligo(dT)	oligodesoxythymidine
PAGE	polyacryloamide gel electrophoresis
PCR	polymerase chain reaction
PD	plasmodesmata
pH	negative decaic logarithm of the proton concentration in mol per litre
PP	phloem protein
Q-TOF	quadrupole- time of flight
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	room temperature
Rubisco	ribulose 1,5-biphosphate carboxylase
s	seconds
SD	standard deviation
SDS	sodium dodecyl sulphate
SE	sieve elements
ssDNA	single strand DNA
SSU	small subunit
Tab.	Table
TEMED	N,N,N,N-tetramethyl ethylene diamine
TFA	trifluoroacetic acid
Tris	2-amino 2-hydroxymethylpropane-1,3-diol
U	unit of enzyme activity (1µmol per 1 min)
UV	ultra violet
v/v	volume per volume
VIS	visible light
w/v	weight per volume
w/w	weight per weight
WT	wild type
x g	times gravity speed

1 Introduction

1.1 Long-distance transport in higher plants

During evolution, plants were successively increasing in size and complexity and reached at one point the critical stage when cell-to-cell transport was insufficient to maintain the physiological control and to satisfy the nutritive demands of all distant plant organs. Upon these demands they had to develop long-distance transport systems that allow the efficient transport of water, nutrients and signalling molecules. In angiosperms two independent cellular conduits, namely xylem and phloem, are mediating long-distance translocation of different substances. The xylem tubes transport mainly mineral-containing water from the roots to the shoots, while the phloem is responsible for the translocation of organic compounds from the sites of synthesis (source organs) to the developing and non-photosynthetic tissues (sink organs). These two transport systems differ not only in their function, but have also different cellular compositions and translocation mechanisms.

1.2 Structure of vascular bundles

The long-distance transport system in higher plants is contained within vascular bundles that are composed mainly of phloem and xylem. Different types of vascular bundles can be distinguished according to the organisation of their components in the cross section view. There are concentric vascular bundles with outside (for example in *Convallaria majalis*) or inside (*Pteridium aquilinum*) xylem, open bi-collateral vascular bundles (*Rannunculus flammula*) and closed collateral vascular bundles (*Zea mais*). Plants from the *Cucurbitaceae* family contain bi-collateral vascular bundles, where the xylem vessels occupy the central part that is surrounded by abaxial and adaxial phloem strands. The central part of stem cross sections of *Cucurbita maxima* (*Cucurbitaceae*) contains an additional pith cavity, and vascular bundles form an outside ring around this cavity. Stems of *Cucumis sativus*, another member of the *Cucurbitaceae* family, do not contain a central cavity, and vascular bundles are symmetrically spread over the complete cross section. Vascular bundles in *Brassica napus* (*Brassicaceae* family) are arranged circularly in the stem and are composed, like in most dicots, of collateral vascular bundles.

1.3 Differentiation of conducting phloem and xylem elements

The functional conduits of the xylem are comprised of dead tracheary elements that degenerate as a result of programmed cell death. During xylem differentiation these cells loose their nucleus and the complete protoplast resulting in the formation of empty, dead and lignified cells. Phloem development similarly incorporates some components of the programmed cell death pathway to produce sieve elements (SE). The functional unit in the phloem is the sieve element-companion cell-complex, within which the individual cells originate from a common mother cell. The two daughter cells formed after the division of this mother cell undergo different developmental programs. During maturation, one of the daughter cells looses its nucleus, ribosomes and vacuoles, resulting in the formation of functional, living SE responsible for long-distance translocation (Oparka and Turgeon, 1999). During their entire lifespan SE depend on the continuous supply of energy and macromolecules from the other of the resulting daughter cells, the companion cell (CC) (Schobert et al., 1998). CC have small vacuoles and are densely packed with ribosomes and mitochondria. The specialised cell-to-cell transport between CC and SE occurs via plasmodesmata (PD) (Oparka and Turgeon, 1999; van Bel et al., 2002) and allows the selective import not only of small substances but also of macromolecules such as proteins and nucleic acids from CC into SE (Ayre et al., 2003). The mature PD between SE and CC are branched on the side of the companion cell that turn into a single pore at the SE side (Oparka and Turgeon, 1999; Ayre et al., 2003). Their frequency (amount of plasmodesmata per complete cell wall) can be higher than 1000. The size of molecules that can be translocated through PD is described by the size exclusion limit (SEL). The SEL for CC-SE plasmodesmata was first estimated to be in the range of 20-40 kDa using fluorescence-tagged macromolecules (Kempers and van Bel, 1997) and the observation that the 27 kDa green fluorescent protein (GFP) moves from CC to the SE and migrates within the sieve tubes of *Arabidopsis* and tobacco (Imlau et al., 1999) confirmed this size range. It was recently demonstrated, using GFP-fusion proteins expressed a under companion cell specific promoter (Stadler et al., 2005), that non-specific trafficking of proteins larger than 65 kDa into sieve elements can occur. Moreover, the SEL of mesophyll PD can be increased by phloem sap proteins from the usual 1 kDa up to 30 kDa (Balachandran et al., 1997;

Ishiwatari et al., 1998). It is therefore conceivable that the large SEL of CC-SE plasmodesmata is caused by the permanent presence of gating effectors in the SE sap.

Neighbouring SE are connected at their end walls by cell wall derived structures called sieve plates. Sieve plates contain sieve pores that allow a continuous passage of nutrients and macromolecules within the SE tube system. Sieve pores derive from modified PD and have with 200-400 nm a much larger diameter than usual plasmatic connections between surrounding cells (Sitte et al., 1998).

The organelles that can be found in mature SE (mitochondria, plastids, endoplasmic reticulum) appear to be anchored to a membrane system that probably also protects some of the SE proteins from the bulk flow (Ehlers et al., 2000). The ER in SE is modified and is proposed to function as a rail system for the trafficking of proteins between SE and CC (Oparka and Turgeon, 1999) and may also take part in protein sorting inside the SE (van Bel and Knoblauch, 2000). The SE plastids are classified into two types. The S-type plastids contain only starch inclusions while P-type plastids enclose protein and starch bodies. The proposed role of the SE plastids is the involvement in the wound-induced occlusion of sieve plates but they may also function as storage units. However, there is no strong evidence for their *in vivo* function so far (van Bel, 2003).

1.4 Mechanisms of loading and transport in xylem and phloem

Xylem sap not only contains mineral salts and water but also some amino acids, organic acids, sugars and several proteins that are taken up or produced by the roots and subsequently distributed to aerial parts of the plant (Lopez-Millan et al., 2000; Buhtz et al., 2004). This translocation system exploits the physical forces produced by water potential differences in the soil-plant-air continuum. Substances are transported in the xylem through the dead and hollow xylem vessels that belong to the apoplasmic space. Minerals that are present in the xylem enter the root mainly by active transport into the symplasm of epidermal cells and move toward and into the stele through PD. They reach the water in the xylem from the cells of the pericycle and parenchyma most likely via active transport. Other substances translocated within the xylem stream are also speculated to be actively transported into the apoplasm before entering the xylem (De Boer and Volkov, 2003).

The phloem transports mainly the products of photosynthesis (carbohydrates and amino acids) from their production sites to photosynthetically inactive organs like flowers, roots, fruits or seeds. Interestingly, storage organs can function as sink or source tissues, depending on the developmental stage of the plant. The photosynthetic products are believed to move within the SE with the mass flow. As the driving force for mass flow, according to the Muench concept (Muench, 1930), the phloem makes use of a turgor gradient along the sieve tubes. High turgor values are formed by the massive photosynthate accumulation occurring within the collection phloem in source tissues that drives water into the SE, while at the sink ends, in the release phloem, low turgor values are generated by the unloading of photosynthates and the subsequent loss of water.

Sugars serve as the primary energy source to generate ATP. Therefore they are used by all cells for storage (in the form of starch), as a carbon source for biosynthesis of most metabolites, and as molecules involved in signal transduction. Some plant species use sucrose derivatives such as raffinose, stachyose and verbascose for transport. However, the major transport form of carbon assimilates in most plant species is sucrose. This non-reducing disaccharide is composed of glucose and fructose. As a non-reducing sugar, sucrose is less reactive than glucose or fructose and as a disaccharide it is more efficient in energy transport than monosaccharides, since at the same osmotic conditions sucrose transports the twofold amount of carbon atoms (double amount of energy) in comparison to the primary photosynthetic products glucose or fructose.

There are two different main strategies for the loading of sugars into the phloem, apo- and symplasmic loading.

Plants that load from the apoplasm and transport sucrose as a major sugar are called apoplasmic phloem loaders (Sjölund, 1997). Among many other plant families e.g. *Asteraceae*, *Fabaceae*, *Poaceae* and also *Brassicaceae* belong to the apoplasmic phloem loaders.

Here, the synthesised sucrose is first transported symplasmically between leaf mesophyll cells via plasmodesmata and is later released into the apoplasm. This efflux occurs by an unknown mechanism probably close to the sites where loading into the SE-CC complex takes place. The uptake of different metabolites into the SE/CC complex is carried out by specific transporters. Sucrose, for example, is actively loaded into the phloem against a concentration gradient in an energy

consuming process by H^+-sucrose cotransporters (Komor et al., 1977). Different sucrose transporters that are CC or SE membrane specific have been described to have a similar function (sucrose import into the phloem) (Lalonde et al., 2003; Lalonde et al., 2004).

In the symplasmic phloem loading process sugars are imported into the phloem through PD. This type of loading is characteristic for tropical plant species but also occurs in several trees and bushes. Also the *Cucurbitaceae* belong to the symplasmic phloem loaders (Oparka and Turgeon, 1999). Symplasmically loading plants transport mainly raffinose and raffinose-like oligosaccharides as well as sugar alcohols (Voitsekhovskaya et al., 2002). The special types of companion cells, called intermediate cells, that contain a number of plasmodesmata are responsible for transport of these sugars into the phloem.

1.5 Sampling of phloem and xylem sap

One major problem for the estimation of phloem and xylem sap components is to obtain pure samples that are free from contaminations from surrounding cells introduced by sampling. The easiest method for phloem sap sampling is spontaneous exudation occurring at incisions in the bark of trees like for example Tree Tobacco (*Nicotiana glauca*) (Zimmermann, 1957; Hocking, 1980) or from detached stems or plant organs in several plants from the *Cucurbitaceae* family (Crafts, 1932; Richardson and Baker, 1982; Kehr et al., 1999; Haebel and Kehr, 2001). Using both methods, phloem exudes only for a short time period and phloem samples can be contaminated by the content of surrounding cells released during cutting (Ziegler, 1975). For several plant species like *Ricinus*, *Lupinus* or *Yucca,* the exudation can last longer so that larger sample volumes can be obtained. Another method for phloem sampling is the exudation into buffer containing chelating and reducing agents that block the closing mechanisms in the phloem, allowing longer exudation times (King and Zeevart, 1974; Tully and Hanson, 1979). Also with this method the risk of tissue damage and contamination from surrounding cells is high (Hanson and Cohen, 1985). The purest phloem samples can be obtained by cutting the stylets of phloem-feeding insects like aphids (Fisher and Frame, 1984). Aphid stylectomy was successfully applied mainly to monocots (Fisher and Frame, 1984; Fisher et al., 1992; Pritchard, 1996) but also several dicotyledonous species like

Brassicaceae have been sampled with this technique (Lohaus et al., 2000; Lohaus and Moellers, 2000). Limiting for this method is the selection of compatible plant-insect combinations, the cutting of stylets and the analysis of the minute amounts of samples that can be obtained (Hayashi and Chino, 1986). In a further method, phloem sap is collected with glass micro capillaries that are inserted into fluorescence-labelled SE under microscopic observation (Raps et al., 2001). In some plant species the collected sample amounts are in the microliter range what allows further analyses (Raps et al., 2001). As an easier alternative to this method, phloem samples can be obtained from small incisions made with sterile needles and can be subsequently collected with a pipette. This method, however, could be successfully applied to a limited number of plant species mainly from the *Cucurbitaceae* family so far (Walz et al., 2004).

Relatively pure xylem sap can be obtained after cutting stems few centimetres above soil level. Surfaces on the root side are first washed with water to remove contaminations from exuding phloem and other cut cells and are subsequently dried with a filter paper. The exuding xylem fluid can be collected with a pipette. Further significant contaminations by phloem sap are excluded because phloem transport occurs from the shoots to the roots while xylem flows in the opposite direction, from the roots into the shoots. This easy method of xylem sampling can be successfully applied to many plant species (Buhtz et al., 2004).

1.6 Composition of phloem and xylem sap

The analyses of phloem samples collected with all the different methods described above show that sucrose is the dominating substance in the phloem of most plant species. Some plants, however, mainly transport different oligosaccharides like sugars from the raffinose family and sugar alcohols (Ziegler, 1975). Besides the transported sugars, also a number of inorganic ions and organic compounds can be found in the SE sap. Within the organic compounds amino acids, organic acids, phytohormones and proteins can be distinguished (Richardson and Baker, 1982; Kallarackal et al., 1989; Hayashi and Chino, 1990). Additionally, the presence of nucleic acids in phloem sap has been previously described (Kollmann et al., 1970; Kühn et al., 1996).

The xylem transports mainly water and mineral salts and therefore the major compound class found in xylem sap is the group of inorganic ions. A large number of different cations and anions could be measured in xylem sap where potassium, nitrate and calcium are dominating (Schurr and Schulze, 1995). The pH values of xylem sap are slightly acidic in the range of 5.5-6.5 (Schurr and Schulze, 1995) while the pH of phloem sap is normally neutral to basic 7-9.

1.6.1 Metabolites

As previously described, phloem sap contains a wide range of metabolites (Richardson and Baker, 1982; Lohaus et al., 2000; Lohaus and Moellers, 2000; Arlt et al., 2001; Fiehn, 2003) that can be transported over long distances or can be responsible for maintaining the functionality of the phloem.

As discussed above, sugars are a major component of phloem sap and the composition of sugars varies between apo- and symplasmically loading species. The majority of plants are apoplasmic phloem loaders and transport mainly sucrose. The concentration of this disaccharide in phloem sap is in the range of 200 - 1400 mM (Lohaus et al., 1995; Marschner, 1995; Lohaus and Moellers, 2000) depending on the plant species. In the phloem of symplasmic loaders like *Cucurbitaceae,* raffinose family oligosaccharides are the dominating sugars with concentrations being in the range of several hundred mM (Haritatos et al., 1996; Knop et al., 2001). In xylem sap, sugars like sucrose are present at very low concentrations being hardly detectable (Satoh et al., 1992; Schurr and Schulze, 1995).

Another abundant group of metabolites in SE sap are amino acids. Their concentration and composition varies between species and is in the range of 5-30 mg/ml in *Cucurbitaceae* (Richardson and Baker, 1982; Arlt et al., 2001), approx. 50 mg/ml in *Ricinus* (Kallarackal et al., 1989), 0.2-0.5 mM in *Brassicaceae* (Lohaus and Moellers, 2000) and 0.04-118 mM in maize (Lohaus et al., 1998). For most of the studied species the major amino acids in phloem sap are glutamine, glutamate, asparagine and aspartate (Richardson and Baker, 1982; Lohaus et al., 1998; Lohaus and Moellers, 2000; Fiehn, 2003).

The amino acid concentration in xylem sap is usually several times smaller than in the phloem. For example in *Nicotiana glauca,* amino acid concentration in phloem sap is 83 mM whereas in xylem sap it is only 2.2 mM (Buchanan et al., 2000).

Several studies (Hall and Baker, 1972; Ziegler, 1975; Richardson et al., 1982; Fiehn, 2003) describe the additional presence of many organic and inorganic acids within the phloem of different species. Measured concentrations of organic acids in *Ricinus* were 2.0-3.2 mg/ml (Hall and Baker, 1972) and approx. 6.7 mg/ml in *Cucurbitaceae* (Richardson and Baker, 1982) and their composition differs between plant species. Additionally, large amounts of phosphate have been detected and from the pool of organic acids malate, citrate and succinate are usually most abundant (Ziegler, 1975; Richardson and Baker, 1982; Fiehn, 2003).

Several organic acids, mainly succinate, malate and citrate could be detected also in xylem sap in low amounts. Additionally the phytohormone abscisic acid is transported through the xylem, mediating stress signalling from the roots into the different parts of the shoots regulating water supply and leaf turgor pressure (Hartung et al., 2002).

Also in the phloem the occurrence of some vitamins, phytohormones and high concentrations of ATP has been previously described (Ziegler, 1975; Sitte et al., 1998).

1.6.2 Proteins

Although transcription and translation can probably not take place in enucleate mature sieve elements (Cronshaw, 1981), their lifetime is usually relatively long (Raven, 1991). The proteins that are needed to maintain functional SE therefore have to be transported into the SE, most probably from the companion cells. It has been shown that along the way between source and sink a steady protein exchange between sieve elements and companion cells occurs (Fisher et al., 1992; Nakamura et al., 1993). The protein composition and concentration in the phloem differ significantly between plant species. The protein content in non-cucurbit species is in the range of 0.1 – 2 mg/ml (Hall and Baker, 1972; Ziegler, 1975; Fisher et al., 1992) while in *Cucurbitaceae* protein concentrations can be even higher than 20mg/ml (Eschrich et al., 1971). The occurrence of structural proteins and protein aggregates in the phloem is known since a long time (Esau and Cronshaw, 1967; Cronshaw and Esau, 1968). Phloem protein 1 (PP1) together with phloem protein 2 (PP2) were described to form filaments in the phloem. PP1 monomers can build soluble polymers via disulfide bridges (Sabnis and Hart, 1979; Read and Northcote, 1983) and further polymerisation under oxidative conditions leads to the formation of

insoluble gel-like structures (Read and Northcote, 1983). This mechanism seems to play an important role in the plugging of SE upon disruption (Alosi, 1988). PP2 proteins belong to the group of lectins. Through the interactions with glycolipid- or glycoprotein- receptors, PP2 can therefore anchor the PP1 filaments to the cellular membrane (Smith et al., 1987).

Besides these structural proteins also soluble proteins are present in phloem sap (Fisher et al., 1992). In SE exudates from several plant species different enzyme activities were measured (Kennecke et al., 1971; Eschrich and Heyser, 1975; Geigenberger et al., 1993; Nakamura et al., 1993; Walz et al., 2002; Hancock et al., 2003) and more than 200 soluble proteins could be detected (Fisher et al., 1992; Nakamura et al., 1993; Sakuth et al., 1993; Ishiwatari et al., 1995; Schobert et al., 1998; Kehr et al., 1999; Walz et al., 2004). A number of these proteins could be identified so far and can be divided into several functional categories. A large group of proteins probably involved in maintaining basic cellular processes inside SE (ubiquitin, heat shock proteins, thioredoxin h) was identified (Schobert et al., 1995). Furthermore, proteins that could generate and amplify signals but also could act as transported signals themselves (systemin, protein kinases) were detected (Nakamura et al., 1993; Ishiwatari et al., 1995; Avdiushko et al., 1997; Yoo et al., 2000; Yoo et al., 2002). Many proteins can also be classified as defence and stress response related (e.g. proteins from the antioxidant defence system and protease inhibitors) (Ryan, 2000; Yoo et al., 2000; Walz et al., 2002; Walz et al., 2004).

Additionally, recently several proteins with RNA-binding properties were also identified in the SE exudates (Xoconostle-Cazares et al., 1999; Barnes et al., 2004) and were postulated to function in signal translocation.

However, the majority of the proteins in the phloem sap remained unidentified and functionally uncharacterised. Most of the collected information about phloem proteins comes from *Ricinus* and *Cucurbitaceae* (Barnes et al., 2004; Walz et al., 2004) where sap collection is relatively easy but protein characterisation in these species remains difficult because their genomes are poorly characterised.

Despite of the fact that the xylem is composed of dead tracheary elements, different proteins were detected in the xylem sap of several plants like apple or peach (Biles and Avbeles, 1991) but most information about xylem polypeptides comes from *Cucurbitaceae* and *Brassicaceae* species (Satoh et al., 1992; Masuda et al., 1999; Buhtz et al., 2004; Kehr et al., 2005). Measured protein concentrations in xylem sap

of different species are in the range of 0.05- 0.1 mg/ml (Buhtz et al., 2004). Most of the identified proteins could function in repair and defence reactions. Some of the most abundant xylem sap proteins like chitinases, peroxidases and serine proteases are common sap components in several plant species (Biles and Avbeles, 1991; Satoh et al., 1992; Buhtz et al., 2004; Kehr et al., 2005). Defence proteins could move with the xylem sap effectively and rapidly to the site of their destination. These proteins may reach xylem sap specifically or could originate from the developing tracheary elements but most likely they are synthesised in xylem parenchyma cells in the roots (Biles and Avbeles, 1991). From the root cells they can be actively secreted into xylem sap (Biles and Avbeles, 1991). For this transition they need amino-terminal target sequences allowing apoplasmic secretion (Nielsen et al., 1997). This type of transport has been previously demonstrated for XSP30 (Masuda et al., 1999) and two glycine-rich xylem sap proteins (Sakuta and Satoh, 2000), and amino-terminal signal peptides have been detected in the sequences of most of the so far known xylem sap proteins (Masuda et al., 1999; Sakuta and Satoh, 2000; Buhtz et al., 2004; Kehr et al., 2005).

1.6.3 Nucleic acids

Nucleic acids have been studied in the phloem since many years {Kollman, 1970 #243}, but significant progress in this topic has been made not until the last decade (Jorgensen et al., 1998; Sasaki et al., 1998; Sitte et al., 1998; Thompson and Schulz, 1999; Xoconostle-Cazares et al., 1999). Three types of nucleic acids are postulated to move over long distances in the phloem: (i) viral genomes, (ii) mRNAs, and (iii) miRNAs.

It has been demonstrated by a number of experiments that viruses can spread their RNA within plants over long distances through the phloem (Lucas and Gilbertson, 1994; Carrington et al., 1996; Goshroy et al., 1997; Roberts et al., 1997; Gough et al., 1999; Ryabov et al., 1999; Thompson and Schulz, 1999; Lucas et al., 2001). This transport is most probably mediated by proteins, since viral encoded movement proteins as well as plant homeodomain proteins can selectively transport single stranded nucleic acids through plasmodesmata into SE (Fujiwara et al., 1993; Lucas et al., 1995).

Besides these viral nucleic acids, also mRNAs have been found in SE exudates. Several mRNAs coding for phloem proteins have been detected in rice by Sasaki *et al.* (1998) using amplification of specific fragments by RT-PCR. Among other mRNAs also the transcripts of sucrose transporter 1 were detected by Knop *et al.* (2004) in phloem exudates of *Alonsa meridionalis*. Like the viral RNAs, also the endogenous nucleic acid molecules are most likely transported as ribonucleoprotein complexes through plasmodesmata into the SE. Phloem proteins that are able to translocate their own transcripts from CC into SE (CmPP16-1 and -2) have been recently identified by Xoconostle-Cazares *et al.* (1999). In addition, other abundant phloem proteins from the *Cucurbitaceae*, the phloem lectins, were shown to have RNA binding activity and were suggested to be involved in long-distance transport of RNA (Gomez et al., 2005). For example, complexes between PP2 and Hop stunt viroid RNA have been documented to occur in the phloem of cucumber (Gomez and Pallas, 2001). Transcripts in the phloem are discussed as a part of the mechanism used by higher plants to integrate developmental and physiological processes (Jorgensen et al., 1998; Thompson and Schulz, 1999). Supporting this suggestion Ruiz-Medrano *et al.* (1999) identified CmNACP mRNA in the phloem that contains a NAC domain that can function in the transcriptional control of apical meristem development. However, the function of mRNAs in the phloem remains controversial and it can not be fully excluded that the detected transcripts may be non-specific spill over or substrates for subsequent degradation and recycling in sink tissues.

Finally, the presence of a number of small interfering RNAs (siRNA) and microRNAs (21-26nt nucleic acid long) in phloem sap has recently been demonstrated by Yoo *et al.* (2004). Additionally, a protein (CmPSRP1) that can bind and transport short 25nt RNA molecules has been found in *C. maxima* phloem exudates (Yoo et al., 2004) and its homologues also occur in other plant species. The role of these small RNAs as signals that negatively regulate gene expression needs further verification but seems to be very likely concerning the high number of detected microRNAs and siRNAs, as well as the identification of possible targets (Yoo et al., 2004) and proteins responsible for their transport.

1.7 Aims and objectives of the present work

The major aim of the present thesis was to identify new soluble phloem proteins in *Cucurbitaceae* and *Brassicaceae* species.

Before *Brassica napus* phloem proteins could be analysed the sample origin and purity should be verified by western blot and sugar concentration measurements followed by metabolic characterisation of the phloem samples with GC MS.

One experimental approach towards protein analyses was to clone phloem protein-coding genes. For this purpose expression libraries from *C. maxima*, *C. sativus* and *B. napus* should be constructed and screened with prepared antisera specific for phloem proteins of the investigated species.

As a complementary approach, some new phloem proteins from *B. napus* should be identified by two-dimensional gel electrophoresis followed by mass spectrometric analysis, within the framework of a comprehensive characterisation of the *B. napus* phloem proteome.

Finally it was planned to initiate studying the possible functions of some of the newly identified candidate proteins using *Arabidopsis* knock out mutants. To allow an investigation of the transportability of the selected proteins in the phloem, grafting of *Arabidopsis* plants should be employed.

2 Materials and methods

2.1 Enzymes and chemicals

Biochemical enzymes and substrates were purchased from Roche (Mannheim) and Sigma (München), chemicals were obtained from Roche (Mannheim), Merck (Darmstadt), Sigma (München). Reagents for SDS-PAGE were from BioRad (München). Radiochemicals were from Amersham-Pharmacia (Braunschweig). Nitrocellulose was from Schleicher-Schuell (Dassel, Germany). Most enzymes were grade II and chemicals were of analytical purity.

2.2 Plant material and growth

Seeds of *Cucurbita maxima* Duch. Cv. “Gelber Zenter” and *Cucumis sativus* L. cv. “Hoffmanns Giganta“ (Treppens, Berlin, Germany) were grown in a phytochamber (16 h light, 8 h darkness; 22℃ daytime, 18℃ night tim e temperature; 60 % relative humidity; light intensity 250 µmol m²s-1). *Brassica napus* L. cv. “Drakkar” (Serasem GIE, la Chapelle d’Armentiers, France) plants were grown in a greenhouse in sterilized soil (Einheitserde® Typ T) under controlled conditions (16 h light, 8 h darkness; 25℃ daytime, 20℃ night time temperature; 55 % relative humidity) watered automatically three times a day with tap water containing fertilizer (Hakaphos® spezial).

Arabidopsis thaliana cv. Columbia 0 (wild type) and knock out mutant lines (glycine-rich RNA-binding protein GRP, annexin 1 and 2, myrosinase) were grown in the same long day phytochamber (16 h light, 8 h darkness; 20℃ daytime, 16℃ night time temperature; 60 - 75% relative humidity). Lines were randomly mixed in the trays.

2.3 Sampling and sample preparation

Phloem exudates from cucurbits and *B. napus* were obtained by wounding 6 - 8 or 12 week old adult plants, respectively, with a sterile needle. In cucurbits, samples were collected from the third internode of the stem. *B. napus* plants were wounded and the

phloem was collected at inflorescence stems, close to the emerging flowers. Initial droplets were discarded and the subsequently exuding phloem sap was collected using plastic micropipettes (Eppendorf, Hamburg, Germany). Sample volumes from one wounding site varied between 1-200 µl for cucurbits and 10-200 µl for *B. napus*.

For one dimensional gel electrophoresis (1 DE), exudates of *C. maxima* and *C. sativus* were expelled directly into 1 DE SDS PAGE sample buffer consisting of 50 mM Tris, adjusted to pH 8.0 with HCl, 1 mM EDTA, 2.5% (w/v) SDS, 5% (w/v) mercaptoethanol, 15% (v/v) glycerol and 0.005% (w/v) bromophenol blue, at a ratio of 1:15. SE exudates from *B. napus* were collected into ice cold proteinase inhibitor cocktail (PIC) (Sigma, Germany) (1 µl PIC/ 100 µl phloem exudate) and then mixed with 1 DE sample buffer.

For high resolution two dimensional gel electrophoresis (2 DE), *B. napus* phloem sap was collected directly into ice-cold acetone/methanol/DTT (90% (v/v), 10% (v/v), 10 mM) and proteins were precipitated over night at -20°C. After centrifugation (15 min, 12000 x g) the protein pellet was washed twice with acetone and air dried for 10-15 min. Proteins were afterwards resolved in 50 µl first dimension buffer containing 2 M thiourea, 7 M urea, 10 mM dithiothreitol (DTT), 4% (w/v) 3-[(3- chloamidopropyl)-dimethylammonio]- propanesulfonate (CHAPS).

To obtain total protein extracts, leaves or inflorescence stems were ground in liquid nitrogen, incubated in 50 mM phosphate buffer (pH 7.0) for 15 min on ice and centrifuged at 14000 rpm at 4℃ for 10 min. Consistent amounts of supernatant containing soluble proteins were mixed with SDS PAGE sample buffer. About 10 µg of protein was loaded on 12% SDS PAGE protein gels.

For several experiments phloem sap was concentrated on 3 or 10 kDa Microcon columns (Milipore, Eschborn, Germany). Up to 500 µl of phloem were loaded onto the column and centrifuged for several hours at 4℃, 14000 x g, until 100 µl solution was remaining on the column. The flow-through fraction was discarded and 100-200 µl 20 mM Na-phosphate buffer was added to the sample on top of the column and centrifuged for further 1-2 hours until about 50 µl of the sample was still above the membrane. Concentrated and purified samples were stored at -20℃ until use.

2.4 Assay of sucrose, glucose and fructose

Frozen stem material was extracted in two subsequent steps with 80% (v/v) ethanol (250 µl per approx. 20 mg FW and 150 µl per approx. 20 mg FW), mixed for 20 min at 80°C and centrifuged at max speed for 5 min. The pellet was re-extracted with 50% (v/v) ethanol (250 µl per approx. 20mg FW) the supernatants were combined and used for immediate assays or stored at -20°C until use. Phloem sap (10 µl) was directly added to 490 µl 100 mM Hepes (pH 7.0) buffer.

Sucrose, glucose and fructose were determined enzymatically in extracts following the protocol of Galtier *et al.* (1993) using a microplate spectrophotometer. The assay contained in a final volume of 250 µl: 169 µl of 100 mM Hepes/KOH with 3 mM $MgCl_2$ (pH 7), 10 µl of 12 mg/ml NADP, 10 µl of 20 mg/ml ATP, 0.5 U glucose 6-phophate dehydrogenase and 50 µl of ethanol extract or diluted phloem. The reactions were started by the successive addition of 1 U hexokinase, 1 U phosphoglucoisomerase and 20 U invertase (all enzymes from yeast).

2.5 GC/MS (gas chromatography/ mass spectrometry) analysis

The polar metabolite fraction was obtained from *B. napus* stem extracts and phloem sap. Samples were harvested in the middle of the day. The stem tissue was frozen in liquid nitrogen and homogenised. 5 µl of phloem sap was mixed with 100 µl of deionised water. Approximately 60 mg of homogenised stem tissue was extracted by vortexing in 300 µl 100% methanol (precooled to -20 °C). Next, 30 µl of internal standard mixture (0.2 mg ribitol, 1 mg d4-alanine, 0.5 mg isoascorbate in 1 ml water) was added to each sample and incubated for 15 min at 70°C shaking. Samples were next vigorously mixed with 200 µl chloroform (5 min, 37 °C) and vortexed subsequently with 400 µl of water and centrifuged at 14000 rpm for 5 min. Two aliquots of the methanol/water supernatants (80 µl) were dried in a speed vac for 6-16 hours.

The dried pellets were redissolved and derivatised with 40 µl of 20 mg/ml methoxyamine hydrochloride in pyridine for 1.5 h at 30°C. Next, retention time standard mixture (10 µl) was added to the samples. The retention time standard mixture contained heptanoic, nonanoic, undecanoic, tridecanoic and pentadecanoic acid 3.7% (w/v) each, tricosanoic and nonadecanoic acid 7.4% (w/v) each,

heptacosanoic acid 22.2% (w/v) and hentriacontanoic acid 55.5% (w/v) in tetrahydrofuran at 10 mg/ml total concentration. The samples were then derivatised by a treatment with 70 µl MSTFA for 30 min at 37°C.

All GC/MS analyses were carried out using a GC/MS system consisting of an AS 2000 autosampler, a GC 8000 gas chromatograph and a Voyager quadrupole mass spectrometer (ThermoQuest, Manchester, GB). The mass spectrometer was tuned according to the manufacturers` recommendations using tris-(perfluorobutyl)-amine (CF43). Gas chromatography was performed on a 30 m SPB-50 comlumn with 0.25 mm inner diameter and 0.25 µm film thickness (Supelco, Belfonte, CA). Injection temperature was 230°C, the interface set to 250°C and the ion source adjusted to 200°C. The carrier gas used was helium set at a constant flow rate of 1 ml/min. The temperature program was 5 min isothermal heating at 70°C, followed by a 5°C/min oven temperature ramp to 310°C and a final 1 min heating at 310°C. The system was then temperature equilibrated for 6 min at 70°C prior to injection of the next sample. Mass spectra were recorded at 2 scan/s with a m/z 50-600 scanning range.

The chromatograms and mass spectra were evaluated using the Masslab program (ThermoQuest, Manchester, GB). A retention time and mass spectral library for automatic peak quantification of metabolite derivatives was implemented within the Masslab method format. For evaluation of relative values within each chromatogram the peak areas derived from specific ion traces indicative for each analysed compound were normalised to the total peak area derived from all measured metabolites present within the same chromatogram.

At least 5 replicates were measured and standard errors (SE) were determined.

2.6 Spectrophotometrical estimation of protein concentration

Protein content in the samples was determined using the BioRad protein assay kit (BioRad, Munich, Germany). The method is based on unspecific binding of the staining agent coomassie brilliant blue to the cationic and non-polar, hydrophobic sites of proteins. Following this reaction the absorption maximum rises from 465 to 595 nm. Absorbance was measured using a UV-VIS Biophotometer (BioRad) calibrated with BSA.

2.7 Protein separation by 1 DE and 2 DE

For 1 DE, samples were denatured by incubation with 1 DE sample buffer (see above) for 15 min at 75℃. The Precision Plus Protein Standard (Bio Rad) was used as a protein molecular weight marker. Electrophoresis was run on 0.75 mm thick 12% mini gels (8 ml 30% acrylamide/bis- solution (Bio Rad), 5 ml 1.5 M Tris/HCl (pH 8,8), 0.1 ml 10% SDS, 6.7 ml H_2O, 0.1 ml 10% ammonium persulphate (APS), 10 µl TEMED). After loading on the gel, samples were separated at constant voltage (80 V) for 2 hours in a Mini Protean 3 Electrophoresis System (Bio Rad) in a buffer solution consisting of 50 mM Tris/HCl, 380 mM glycine, 0.1% (w/v) SDS, pH 8.6. Proteins in the gels were visualised either by staining with colloidal Coomassie blue (Invitrogen, Karlsruhe, Germany) over night, according to the supplier instructions, followed by destaining in distilled water or by silver staining using the Silver-Stain-Plus-Kit (BioRad) according to the protocol of the supplier.

High resolution 2 DE was performed together with Patrick Giavalisco (AG Kehr, Max-Planck Institute, Golm, Germany) and Berit Ebert (AG Fisahn, Max-Planck Institute, Golm, Germany) according to the protocol from Giavalisco *et al.* (2003). Isoelectric focusing (IEF) was run on 16 cm long, 1.5 cm thick tube gels and 200 µg or 100 µg protein were applied to the first dimension.

After IEF the gel stripes were subsequently equilibrated for 15 min in buffer 1 solution (6 M urea, 2% SDS, 0.375 M Tris/HCl pH 8.8, 20% glycerol, 2% (w/v) DTT), and buffer 2 solution (6 M urea, 2% SDS, 0.375 M Tris/HCl pH 8.8, 20% glycerol, 2.5% (w/v) iodoacetamide) and applied to 12% SDS polyacrylamide gels (18/16/0.1 cm) in the second dimension.

Proteins on 2 DE gels were stained with coomassie blue according to Doherty *et al.* (1998).

2.8 Visualisation of glycosylated and phosphorylated proteins

To detect posttranslational modifications, phloem proteins were separated on 12% SDS PAGE gels (10 µg per lane) and 2D PAGE gels (200 µg). These gels were stained for glycoproteins using the Pro-Q Emerald 300 Glycoprotein Gel and Blot Stain Kit (Molecular Probes, Leiden, The Netherlands). The detection is based on the

reaction of a fluorescence dye contained in the staining solution with oxidised carbohydrate groups on the glycoproteins.

Phosphoproteins were visualised using the Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes) following the procedures supplied with the kit. This fluorescent stain allows a direct detection of phosphate groups attached to tyrosine, serine and threonine residues. The Pro-Q Diamond stain has excitation/emission maxima at ~550/580 nm and can be detected with a visible-light transilluminator.

2.9 Anti-phloem protein antibody production

Three different antisera were raised in rabbits against the total phloem protein complements of *Cucumis sativus*, *Cucurbita maxima* and *Brassica napus*. To obtain a sufficient amount of protein (1 mg) for immunisation, phloem sap was collected using two different methodologies. *B. napus* phloem sap was collected directly into Trizol (1 ml phloem sap: 3 ml Trizol) and mixed with 600 µl chloroform/isopropanol (24:1) solution, followed by over night protein precipitation at –20°C with 3 volumes of 100% acetone. For the precipitation of major phloem proteins (PP1 and PP2) from *C. sativus* and *C. maxima,* phloem sap was mixed with 10 mM Tris/HCl-phosphate buffer, pH 4.5 containing 2 mM DTT at a ratio of 1:5, then with 5 µl 2 M HCl followed by 5 µl 2 M NaOH. Over night precipitation of all remaining proteins was carried out using the same procedure as that used for *B. napus* samples. Precipitated proteins were centrifuged and resulting pellets air-dried, washed once with 100% acetone, air-dried again and used for immunisation. Immunisation procedure and antiserum production was performed by BioGenes GmbH, Berlin.

2.10 Western blotting

Immunoblotting was performed as described in Buhtz *et al.* (2004). After electrophoresis, gels were incubated for 10 min in standard transfer buffer containing 20% methanol and subsequently, the gel separated proteins were transferred either onto nitrocellulose membranes (0.2 µm Porablot, Schleicher-Schuell, Germany) for 1 DE immunoblots using a mini transblot cell system (BioRad) (1.5 h blotting at 75 V) or onto PVDF membranes under semi-dry conditions using a Trans Blot SD Semi Dry Transfer Cell (BioRad) for 2D-gel immunoblots (1.5 h, 400 mA). Blots were stained

for total protein using amido black (1% (w/v) in 10% (v/v) acetic acid) and destained in water. After blocking with a 3% solution of bovine serum albumin (BSA), blots were incubated with primary antibodies for 2 h. Three steps of washing in TBS-T solution (Tris base 20 mM, NaCl 150 mM, pH 7.4, 0.05% Tween 20) were applied after the incubation with antibodies. For the detection of antibody-bound proteins, secondary antibodies coupled to alkaline phosphatase were used and complexes were visualised by adding NBT/BCIP solution (Roche Diagnostics GmbH, Mannheim, Germany).

Primary antibodies used in the western blots in addition to the phloem protein specific antibodies described above were the following: polyclonal antibodies against purified recombinant tobacco activase were a gift from Prof Steven J. Crafts-Brandner and Dr. Michael E. Salvucci, (USDA-ARS, Phoenix, USA). Antiserum directed against recombinant 14-3-3 protein was kindly provided by Prof. Jan Szopa (University of Wroclaw, Poland). Polyclonal peptide anti-dehydrin antibodies were commercially available (Biomol, Hamburg, Germany). Monoclonal mouse antibodies directed against the small subunit of rubisco were provided by the group of Prof. Micheel (University of Potsdam, Germany). Anti-rapeseed myrosinase monoclonal mouse antibodies were a gift from Prof. Johan Meijer (Swedish University of Agricultural Science, Uppsala, Sweden). Anti-glycine-rich RNA-binding protein specific peptide antibodies were produced against the peptide IDSKIINDRETGRS by BioGenes.

Secondary antibodies used for the detection of primary antibodies were commercially available anti-rabbit IgG, anti-mouse IgM and anti-rat IgG conjugated to alkaline phosphatase (Sigma, Germany).

2.11 Tissue printing and whole leaf electro blotting

Tissue prints were obtained from transverse sections of young stems from 10 week old cucurbits and inflorescence stems of *B. napus*. Immediately after excision, exposed tissue was blotted to nitrocellulose by applying consistent pressure for 2-4 s repeatedly onto a membrane. Blots were air dried and incubated for two hours in 3% bovine serum albumin blocking solution (Sigma, Germany).

The whole leaf electro blotting procedure was performed as previously described in Terras *et al.* (1998). Cuticular wax from young leaves of *C. sativus* and *C. maxima* plants was removed by rinsing in chloroform for three minutes (Stammitti et al.,

1996). Then leaves were air dried and subsequently soaked in electro blotting transfer buffer (48 mM Tris-HCl, 39 mM glycine, 20% methanol, pH 8.0). The abaxial leaf surface was positioned on the nitrocellulose membrane on the cathode side. A mini transblot cell system (BioRad) was used for tank electro blotting in standard transfer buffer containing 20% methanol (1.5 h blotting at 75 V).

For immunoreactions, primary antibodies against total phloem proteins from *C. sativus* and *C. maxima* were used following the procedure described above.

2.12 Affinity purification of antibodies

To separate the antibodies that react strongly with rape phloem proteins from the complete antiserum, affinity purification was performed. 400 µl of *B. napus* phloem sap were concentrated on 3 kDa Microcon columns (Milipore) according to the protocol of the supplier to 50 µl and eluted with another 50 µl of water. After incubation of the samples with 100 µl 1 DE sample buffer (see above) for 15 min at 75℃, samples were loaded onto 9 separate slots of one 1 DE SDS PAGE mini gel. Electrophoresis conditions were as described above. Proteins were blotted onto a nitrocellulose membrane (as described above) and stained with amido black to visualise protein bands. Nitrocellulose with proteins from one of the 9 slots was cut out and incubated with anti-rape phloem protein antiserum as a control. Three major bands of proteins marked by antibodies were chosen to further processing. Nitrocellulose with proteins representing these three bands (in the size of ~31 kDa, ~28 kDa and ~25 kDa) from each of the remaining slots was cut out and placed in three reaction tubes separating the bands. Each sample was blocked with 3% BSA for 1.5 h, washed with TBS buffer (Tris base 20 mM, NaCl 150 mM, pH 7.4) and incubated with anti-rape phloem antiserum for 2 h. After three washing steps (5 min each) in TBS buffer, antibodies bound to the proteins were subsequently eluted from the membrane. Incubation for 10 min with 1 ml of 0.1 M glycine/HCl solution (pH 2.5) was repeated thrice, and supernatants with eluted antibodies were collected into reaction tubes containing 100 µl 1 M Tris pH 8.0. These eluted antibodies were used as the primary antibodies in western blots with complete rape phloem proteins as described above.

2.13 Immunoprecipitation of proteins

To study the immunogenic epitope, rape phloem proteins that reacted with antiserum were purified by immunoprecipitation and analysed by 1 DE. 1000 µl phloem sap from rape were concentrated 10 times on 3 kDa microcon columns and used for immunoprecipitation experiments. Dynabeads coupled with protein A (Dynal, Germany) were applied according to the supplied protocol. 100 µl of the Dynabead solution were incubated with 10 µl anti-rape phloem protein antiserum for 30 min. After three washing steps with 0.1 M Na-phosphate buffer containing 0.1% Tween 20 (pH 8.1), the bound antibodies were chemically cross-linked to protein A using 20 mM DMP (dimethyl pimelimidate dihydrochloride) solution. Dynabeads with bound antibodies were then incubated with 100 µl of phloem protein extract for 1 h and washed afterwards three times with PBS (phosphate buffered saline) solution. The bound proteins were eluted with 30 µl 0.1 M citric acid (pH 3.1) twice and the antigen-containing supernatant was collected and stored at -20℃ . 10 µl solution of the eluted proteins were applied on 1 DE gel and western blots were performed as described above. Anti-rape phloem protein antiserum was used as the primary antibody.

2.14 Calcium binding experiments

To select the proteins that bind calcium ions from the complete phloem sap, 100 µl of concentrated (3 kDa Microcon columns) SE exudate were applied onto Vivapure Metal Chelate Mini Spin Columns (Vivascience) with immobilised calcium ions. Columns were first pre treated with 400 µl 1.0 M NaCl than twice with 400 µl 0.5 M $CaCl_2$ to bind Ca^{+2} ions and again twice with 400 µl 0.1 M NaCl (centrifugation for 1 min, 5000 x g). Next, the columns were twice equilibrated with 400 µl equilibrating solution 0.05 M NaH_2PO_4 and 0.3 M NaCl pH 8.0 (centrifugation 1 min, 3000 x g) and the sample was clarified on supplied with the kit clarification columns (5 min, 2000 x g). Clarified samples were loaded onto the columns and centrifuged for 3 min at 3000 x g. This step was repeated twice using the flow-through fraction as a sample. After one single washing step (400 µl equilibrating solution, 3 min, 3000 x g), bound proteins were eluted three times with 250 µl elution buffer (250 mM imidazole, 0.1 M EDTA, 0.3 M NaCl, 50 mM NaH_2PO_4) for 3 min at 1500 x g. The eluates were concentrated on Microcon columns and loaded on 1 DE gels.

2.15 cDNA library construction and screening

Expression libraries of *C. sativus* and *C. maxima* were constructed by sub-cloning cDNAs from existing libraries into the vector allowing expression in prokaryotes. A *B. napus* library was constructed from complete rape RNA.

2.15.1 RNA extraction and mRNA isolation

Total RNA was extracted from leaves, stems and inflorescence stems of 6-8 week old *B. napus* plants. Total RNA was isolated from about 2.5 g of tissue according to the Trizol RNA extraction protocol. 5 ml Trizol were added to 0.5 g of frozen and ground tissue samples, vortexed for 30 sec and incubated at room temperature for 5 min. Samples were centrifuged 20 min at 4°C and 4000 rpm, the supernatants were transferred to a new tube and 3 ml pure chloroform were added. The mixture was vortexed and incubated at RT for 5 min. After centrifugation (20 min, 4000 rpm, 4°C), the RNA-containing aqueous phase was transferred to a new tube. 1/10 volume of 3 M sodium acetate and 0.6 volumes of isopropyl alcohol were added, mixed, and incubated at 4°C for 15 min. Afterwards, samples were centrifuged for 10 min at 4000 rpm and 4°C to pellet RNA. The supernatants were removed, pellets were washed twice with 1 ml 70% Ethanol, centrifuged for 5 min at 4000 rpm and 4°C. Finally, the pellets were air dried for 10 min at RT and resuspended in 50 µl DEPC treated water. RNA samples were then further purified with the RNeasy® Plant Mini Kit (Qiagen, Germany) according to the protocol of the supplier. Concentration of RNA was measured spectrophotometrically with a UV-VIS Eppendorf Biophotometer at a wavelength of 260 nm in UV light compatible cuvettes (UVetten, Eppendorf). 1 µl of RNA was diluted with 50 mM Tris/HCl buffer pH 7.4 mixed and measured using buffer as a blank.

Poly(A)+ RNA was isolated from the total RNA pool following the protocol of the supplier (Oligotex mRNA Mini Kit, Qiagen, Germany). Approximately 500 µl of isolated RNA were used for mRNA isolation.

2.15.2 cDNA library construction

5 µg of *B. napus* mRNA was used for cDNA expression library construction with the ZAP Express cDNA Synthesis Kit (Stratagene, Germany) following the manufacturers' instructions. First, cDNA clones containing adapter sequences for restriction enzymes (Eco R I and Xho I) were synthesised. Second strand cDNA was synthesized with ^{32}P radiolabeled dCTP. Inserts were next size fractionated on 1 ml disposable columns packed with CL2B Sepharose to eliminate DNA fragments smaller than 400 bp. Fractions containing inserts above 400 bp were pooled and used for ligation into the ZAP Express vector. To achieve this, 1 µl of purified cDNA was mixed with 1 µl (0.6 µg) ZAP Express vector, 2 U T4 DNA ligase, 0.5 µl 10 mM rATP in the ligase buffer (50 mM Tris/HCl pH 7.5, 7 mM $MgCl_2$, 1 mM DTT- final concentration) and incubated over night at 12 ^oC.

Expression libraries of *C. sativus* and *C. maxima* were constructed by sub-cloning cDNAs from existing libraries (in pCMV SPORT 6.1 vector, Invitrogen, Germany) into the lambda ZAP Express vector (Stratagene, Germany) by NotI/EcoRI directional cloning. For this purpose, 1 µg of plasmid DNA (plasmid library in pCMV SPORT 6.1 vector) was digested with Not I/Eco R I/ Xba I restriction enzymes (1.5 h, 37 ^oC, 10 U of enzyme per reaction) and 5 µg of ZAP Express vector were digested with Not I/Eco R I enzymes. The digested DNA was subsequently purified with 1 volume of a phenol/chloroform mixture, followed by NaOAc and ethanol precipitation (1/10 volumes 3 M NaOAc and 2.5 volumes of ice-cold 100% ethanol). Inserts were ligated into the vector in the ligation reaction according to the Stratagene protocol. Pre-digested ZAP Express vector was incubated with the digested libraries (at a molar vector to insert ratio of 1:1) for 5 min at 65^oC, followed by over night incubation at 16^oC with 2 U T4 DNA ligase, 0.5 µl 10mM rATP in ligase buffer (50 mM Tris/HCl pH 7.5, 7 mM $MgCl_2$, 1 mM DTT).

DNA concentrations were measured spectrophotometrically with a UV-VIS Eppendorf Biophotometer at the wavelength of 260 nm in UV light compatible cuvettes (UVetten, Eppendorf). 1 µl DNA was diluted with 50 mM Tris/HCl buffer pH 7.4 mixed and measured using buffer as a blank.

Inserts from each library cloned into the vector were packed into phage particles using the Gigapack III Gold Packaging Extract (Stratagene) according to the manufacturers' protocol. In short, 2 µl of each ligation reaction were added to the

phage extract and incubated for 2 h followed by the addition of 500 µl SM buffer (5.8 g NaCl, 2 g $MgSO_4$x 7 H_2O, 50 ml of 1 M Tris/HCl pH 7.5, 5 ml of 2% (w/v) gelatine in a final volume of 1 l) and 20 µl chloroform. After short centrifugation the collected supernatants contained the phage particles.

To test the titre and the quality of the libraries, several phage dilutions (10^{-2} to 10^{-4}) were prepared and 1 µl of the phage solution was used to infect 200 µl host bacterial cells (XL1-Blue MRF´) at an OD_{600} of 0.5. Infected cells were plated with 3 ml NZY top agar on IPTG-X-gal containing plates. The colour selection by α-complementation with the ZAP-Express vector requires IPTG and X-gal for generation of a blue colour. Background plaques (not containing an insert) appear blue, while recombinant plaques are white.

The titer of the libraries was estimated by counting the plaques and determining the concentration of the library (plaque forming units pfu/ml) based on the dilutions. The *C. maxima* library resulted in 7 800 000 pfu, the *C. sativus* library in 5 000 000 pfu and the *B. napus* library contained 500 000 pfu.

From each of the plated libraries about 20 plaques were randomly picked and inserts were isolated from the plasmid DNA of bacterial strains (XLOLR, Stratagene, Germany) with incorporated phagemids (see above). Plasmid DNA was then digested with restriction endonucleases that cut the phagemid at the flanking sites of the insert.

2.15.3 Screening of the expression libraries

About 50 000 clones from each library were screened with the respective anti-total phloem protein antiserum (diluted 1:5000) following the instruction manual for immunoscreening supplied with the Stratagene *pico*Blue™ immunoscreening kit. After three rounds of screening, positive clones were selected and insert sizes were verified by restriction endonuclease digestions. In a total volume of 10 µl 1-2 µl of plasmid DNA were digested with 1 U of each of the two restriction enzymes in the appropriate buffer solution diluted with sterile water. The reaction was run for 1.5 hours at 37°C. Complete digestion mixtures were separated on 1% agarose gels containing ethidium bromide.

Fragments larger than 600 bp were sequenced (AGOWA GmbH, Berlin, Germany).

The obtained nucleotide sequences were used for database similarity searches with the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/) against a nucleotide database. Nucleotide sequence alignments were performed with an alignment program (http://www.ch.embnet.org/software/LALIGN_form.html).

2.16 Isolation of CSF2 clones from the plasmid libraries

To verify that the original *C. maxima* and *C. sativus* expression libraries contained known phloem protein-coding clones that can be successfully isolated and sequenced, plasmid libraries of *C. maxima* and *C. sativus* (in pCMV SPORT 6.1 vector) were screened for the occurrence of the transcript of the CSF2 gene. CSF2 proteins have been previously detected in the phloem of cucumber and pumpkin and the *C. sativus* CSF2 nucleotide sequence is known (Haebel and Kehr, 2001; Walz et al., 2004). Libraries were screened with a radiolabeled cDNA probe. For this purpose DNA was isolated from pSK plasmids containing a fragment of the CSF2 cDNA from *C. sativus* (using the method for plasmid DNA isolation described below) and 10 µl of isolated DNA was subsequently digested with Eco R I to cut out the insert from the vector. Digestion reaction contained in total volume of 50 µl 10U of Eco R I, 10 µl of DNA and was run in appropriate buffer solution for 1.5 h at 37 ^{o}C). After digestion, the products were resolved on a 1% agarose gel and fragments in the size of approximately 650bp were cut out from the gel and isolated using a gel-extraction kit (Qiagen) following the supplied protocol. 50 ng of isolated DNA was used for probe synthesis using the *redi*prime^{TM} II kit (Amersham) and ^{32}P radiolabeled dCTP according to the supplied protocol.

Libraries were screened following the protocols in (Sambrook et al., 1989). After three rounds of screening plasmid DNA was isolated from positive bacterial colonies and inserts were cut out with Not I/Eco R I restriction enzymes. Digestion reactions were run on an agarose gel and selected clones were sequenced (AGOWA).

2.17 Plasmid DNA isolation

Plasmid DNA was isolated from XLOLR bacterial strains (Stratagene, Germany) infected prior with phagemids derived from positive clones from the library screening (according to: in vivo mass excision protocol, Stratagene). 1.5 ml from a bacterial

culture grown over night at 37℃ were briefly centrifu ged and the pellet of bacterial cells was resuspended in 100 µl solution 1 (25 mM Tris/HCl, 10 mM EDTA, pH 8.0). Subsequent addition of 200 µl solution 2 (1% SDS, 0.2 M NaOH) and 150 µl of solution 3 (3 M potassium acetate) followed by gentle mixing by inversion caused lysis of the cells. Subsequently, 250 µl of phenol/chloroform/isoamyl alcohol (25/24/1) were added and vortexed. After 15 min incubation at -20℃ and centrifugation at 14000g for 5 min, the upper aqueous phase was transferred to a new reaction tube and plasmid DNA was precipitated with 1 volume of isopropanol during 15 min incubation on ice. Samples were then centrifuged (15 min, 14000g) and washed with 70% ethanol. DNA pellets were air dried for about 15 min at room temperature and suspended in 30 µl water containing RNAse H (10 mg/ml RNAse H diluted 1:1000 in distilled water).

2.18 Protein mass spectrometry

Protein mass spectrometry (MS) was performed as described in Giavalisco *et al.* (2005).

2.18.1 Trypsin digestion

Protein spots from 2 DE stained with colloidal coomassie were cut out from the gel, placed in 0.5 ml siliconised, nuclease free reaction tubes (Ambion) and destained for 2 h in a solution containing 40% (v/v) acetonitrile and 60% (v/v) NH_4HCO_3. Destained spots were dehydrated in 100% acetonitrile for 5 min and air dried for 5 min. 20 µl of modified trypsin solution (0.001 µg/µl in 50 mM NH_4HCO_3) were added to each sample and after complete absorption of the solution by the gel piece (about 30 min) 30 µl 50 mM NH_4HCO_3 solution were added. The digestion reactions were incubated over night at 37℃. For the elution of the proteolyti c peptides, gel pieces were incubated first with 50 µl 5% (v/v) trifluoroacetic acid (TFA) for 20 min then three times with 50 µl 5% (v/v) trifluoroacetic acid in 50% acetonitrile for 20 min. Digestion supernatants and eluates of each gel piece were collected together into a new reaction tube. Collected samples were dried by vacuum centrifugation.

2.18.2 Desalting and concentrating of tryptically digested peptides

Samples for mass spectrometric analysis were desalted and concentrated with 'reversed-phase' pipette tips (Zip Tips C_{18}, Millipore). At first, samples were solved in 7 µl 0.1% TFA and 3 µl 8 M guanidine in 2.5% (v/v) TFA were added. The Zip Tip pipette tips were prepared by two cycles of filling and emptying with 50% acetonitrile in 0.1% TFA, followed by two cycles with 0.1% TFA solution. Peptides were adsorbed to the reversed-phase material in the tips by 10 cycles of filling and emptying the tip with sample solution. After washing (3 cycles of filling and emptying with 0.1% TFA) the bound peptides were eluted with 3-5 µl 75% acetonitrile, 0.1% acetic acid solution into separate reaction tubes. The eluted samples were directly used for mass spectrometric analyses.

2.18.3 Mass spectrometry

1 µl of each tryptically digested and desalted peptide sample was analysed with a quadrupole-time-of-flight mass spectrometer (Q-TOF MS, Micromass). The instrument was calibrated externally with the fragment spectrum of the doubly charged 1571.68 Da peptide of fibrinopeptide B. Samples were introduced into the instrument by nanoelectrospray with gold-coated borosilicate glass capillaries (Micromass). Capillary and cone voltages were set to 800 V and 50 V, respectively. Collision energy varied from 15 to 35 V depending on mass and charge of the peptides. Tandem MS data were processed using the MaxEnt3 algorithm. Amino acid sequence information was deduced from fragmentation spectra with the PepSeq programme of the Biolynx software (Micromass) with 0.3 mass-to-charge ratio (m/z) tolerance.

Deduced sequences were used for database searches using the BLAST algorithm for short protein sequences available in the internet (http://www.ncbi.nlm.nih.gov/). Database similarity searches were restricted to the green plants (*Viridiplantae*). All the sequences were simultaneously compared (using a freely accessible align program http://www2.igh.cnrs.fr/bin/align-guess.cgi) to trypsin and keratin to exclude possible self digestion fragments of trypsin and keratin contaminations, respectively.

2.19 Screening of Arabidopsis T-DNA mutants

2.19.1 Genomic DNA isolation

One mature leaf from each mutant plant was mashed in a 1.5 ml reaction tube using a plastic stick and next incubated with 300 µl CTAB buffer (2% (w/v) cetyl-trimethyl-ammonium bromide (CTAB) 1.4 M NaCl, 0.1 M Tris/HCl pH 8.0, 20 mM EDTA) for 60 min at 65°C. After the samples cooled down, 300 µl of chloroform was added and the samples were vortexed and centrifuged briefly at 14000 x g. The upper aqueous phases were transferred into a new reaction tube and 300 µl of isopropanol were added to precipitate DNA. After a short centrifugation (5 min, 14000 x g) the pellets were washed twice with 80% ethanol and air dried for 10 min. DNA pellets were resuspended in 100 µl of TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA).

2.19.2 Isolation of homozygous lines

Arabidopsis thaliana mutants and WT plants were tested for the presence of T-DNA inserts by the amplification of specific DNA fragments using polymerase chain reaction (PCR). Two PCR reactions were performed for each mutant line. One reaction detecting a T-DNA insertion within the gene was performed with one primer complementing the T-DNA sequence (Lba 1 or LBb) and the other complementary to the fragment of the gene sequence. Gene specific primers were either complemented to the fragment close to the left (LP) or right border (RP) of the gene. The second reaction was performed to amplify the undisrupted gene and therefore contained the primer pair designed for the gene sequence of interest (LP and RP). When products were amplified in both reactions the line was described as heterozygous, if only one reaction gave a product the line was homozygous or wild type, respectively. 1 µl of genomic DNA was used as a template in all reactions that contained 200 µM of each dNTPs, 0.5 µM of each of the primers, 1.5 mM MgOAc, 1 µl 10x PCR buffer and 0.5 U of Taq DNA polymerase (self made) in 50 µl end volume. 10x concentrated PCR buffer for the Taq DNA polymerase contained 500 mM KOAc, 1% Tween 20, 15 mM MgOAc, 500 mM Tris/HCl pH 8.4). After the first denaturating step (5 min at 94°C) 35 PCR cycles were run (30 sec at 94°C, 60 sec at variable annealing temperatures, 60 sec at 72°C), followed by a final step of 3 min at 72°C. The annealing temperatures were estimated separately for each primer pair.

PCR products were detected by separation of 10 µl of the reaction mixtures on 1% agarose gels containing ethidium bromide.

2.20 Grafting

Grafting of *Arabidopsis* plants was performed as described in Rhee & Somerville (2004). 6-8 weeks old *A. thaliana* plants grown in a phytochamber at long day conditions were grafted. Plants were approximately 10-15 cm in height. To graft, the primary or secondary inflorescence stem of a rootstock was severed horizontally using a razor blade. The rootstock (WT) was inserted approximately 0.5 cm into a capillary tube (1 cm in length with a 1 mm internal diameter) filled with distilled water. The inflorescence stem of a scion (from GRP7, annexin 1 and 2 and myrosinase mutants) was cut horizontally and inserted into the capillary tube so that the two ends were firmly appressed. Grafted plants were kept under 100% air humidity for 3-4 days until the junction was formed. Humidity was decreased gradually over a period of 3 to 4 days. The capillary tubes were not removed during the further growth of the grafted plants.

3 Results

3.1 Sample collection

The work described in the following chapters consists mainly of analyses of phloem sap samples collected from stems of different plant species.

3.1.1 Phloem sampling

It is possible to collect reasonable amounts of phloem sap from cucumber (*C. sativus*) and pumpkin (*C. maxima*) using the modified exudation technique (Walz et al., 2002; Walz et al., 2004). Phloem was obtained by wounding 6-8 weeks old adult cucumber and pumpkin plants at the third internode of the stem using sterile needles. Depending on the plant, between 5 and 20 µl samples could be collected from one wounding site and the exuding phloem sap was expelled directly to the appropriate solution after the first droplet was discarded. The protein concentration in the phloem samples was 30 – 60 µg/µl and enabled subsequent protein analysis.

For *B. napus* phloem sample collection, the modified exudation technique described above was applied on inflorescence stems of 8-10 week old plants. Exudation was restricted to one developmental state, just after flowers started to emerge, and to this single location (Fig. 1). Phloem sap (up to 100 µl from one puncture) was collected after removal of the first droplet to minimise contamination by destroyed cells (Giavalisco et al., 2005). Intensive exudation lasted for about two hours and after that decreased until it stopped. During the period of exudation the protein composition detectable on SDS-PAGE gels remained constant and also the protein concentration was stable in the range of 1 µg/µl.

Fig. 1 Phloem sap sampling from 8 week old B. napus plants. A small incision is made with sterile needle into the inflorescence stem and exuding phloem sap (indicated by an arrow) is collected with a pipette tip.

3.1.2 Sample quality verification

The sampling technique based on exudation used in this work for sampling phloem from cucumber and pumpkin was used and described in the literature before (Walz et al., 2002; Walz et al., 2004). As presented in the PhD thesis of C. Walz, the differences in protein patterns between phloem protein samples and protein extracts from other tissues of cucumber and pumpkin can be easily distinguished already by 1-dimensional SDS-PAGE gel electrophoresis. Additionally no products were amplified when phloem samples from cucumber and pumpkin were tested by RT-PCR for the presence of rubisco (Walz, 2002), indicating that there was no RNA from other cells present in the phloem samples.

To confirm the phloem origin of the samples also in rape, sugar concentrations were measured in the obtained exudates. The most abundant sugar detected was sucrose with concentrations of about 600 mM, while the reducing sugars like glucose and fructose were hardly measurable. Such a sugar composition would be expected for pure phloem samples (Geigenberger et al., 1993). The sampling technique used to probe phloem sap could cause contaminations by collapsing cells close to the sieve elements (SE). The degree of such impurities was assessed by testing for the presence of rubisco protein in the samples. Western blot analysis of samples from different rape tissues, using an antibody directed against the small, nuclear encoded

subunit of rubisco, showed that this protein could not be detected in phloem samples, while it was clearly present in leaves and stem tissue (Fig. 2).

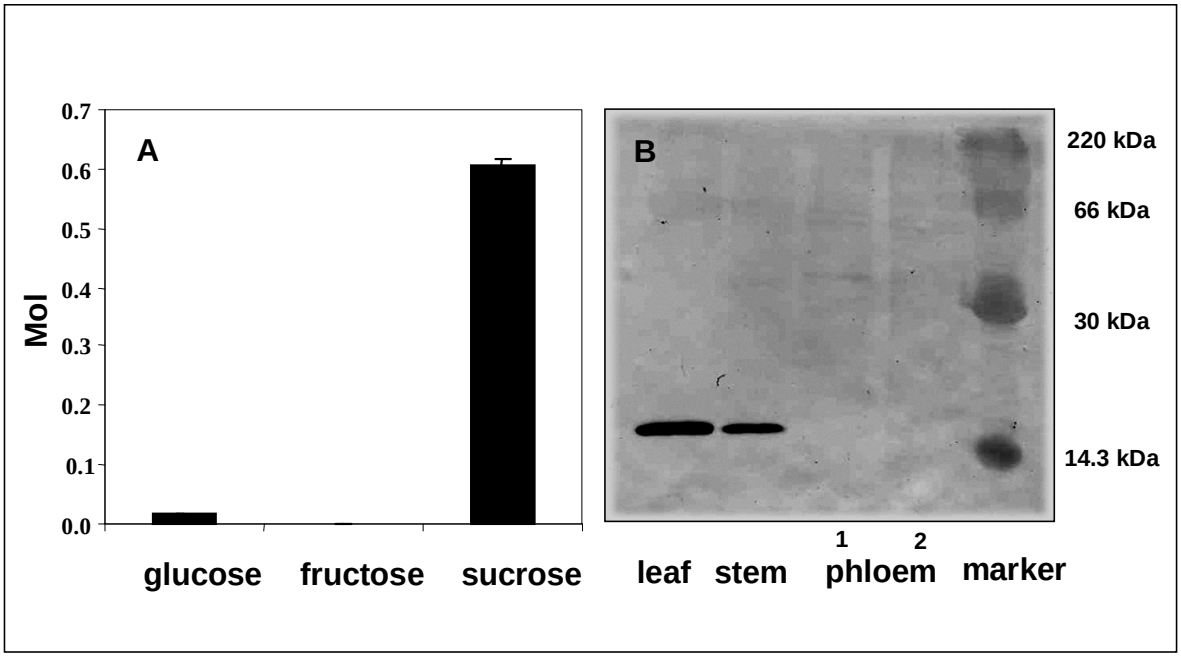

Fig. 2 Concentrations of glucose, fructose and sucrose in rape phloem samples (A) and Western blot analysis of protein extracts from different tissues using monoclonal anti-small subunit of rubisco antibodies (B). The measured concentration of sucrose (~0.6 Mol) in comparison to the very low contents of glucose and fructose indicate that relatively pure phloem samples were obtained. For the western blot tests (B) about 10 µg of protein from leaf and stem extracts and 20 and 10 µg from phloem samples (sample 1 and 2 respectively) were loaded and separated on 1D SDS-PAGE gels. No detection of the small subunit of rubisco was observed in either phloem sample in contrast to leaf and stem protein extracts, where strong signals were found.

3.1.3 Metabolic profiling of phloem sap and stem tissue

3.1.3.1 Composition of metabolites in the phloem

Metabolites in SE exudates of cucurbits have already been widely studied as described by Richardson *et al.* (1982) and Fiehn (2003). To further characterise the phloem samples obtained from *B. napus,* metabolite profiling by GC-MS measurements was performed. The results were compared with the metabolite profiles of stem tissue.

For the analyses, 5 µl of phloem sap was derivatised and introduced into gas-chromatography coupled with a quadrupole mass spectrometer. For the measurements of metabolites in the stem, about 60 mg (fresh weight) tissue was

measured. The resulting chromatograms were processed with the Masslab software and metabolites were identified by their fragmentation spectra. Subsequently, all automatic peak detections were manually checked for false positive and false negative assignments. The mass spectra were then compared to in-house and commercially available mass spectra libraries. Relative amounts were calculated according to the peak area of the substance normalised to the complete peak area of all identified metabolites. The measured metabolites were divided into three groups: amino acids and amines, sugars and sugar alcohols, and a third group composed of organic and inorganic acids. In the phloem sap of rape, 30 substances from the group of amino acids and amines were detected (Fig. 4 A and Table IV in the appendix) with glutamine and valine being the most abundant. Because of the high similarity of the spectra and retention times, arginine and ornithine remained undistinguishable. Among the proteogenic amino acids only histidine was not detected and the remaining 19 aa were dominating over the amines dopamine, spermidine, putrescine, amino acid analogues like GABA and amino acid derivates (S-met-cysteine, O-acetylo-serine, β-alanine). It was also possible to identify 21 different sugars and sugar alcohols. Sucrose was observed on the chromatograms as a massively dominating, overloaded peak that could not be considered for calculations. For this reason, the concentrations of sucrose and additionally of glucose and fructose were measured separately by enzyme assays (Fig. 3). These three sugars were therefore excluded from the following calculations. As it was expected for an apoplasmic phloem loader like rape, the most abundant sugar in the phloem sap was sucrose. Glucose and fructose were detected at very low levels. Due to the similar retention times and fragmentation spectra it was impossible to distinguish between two alcohols: sorbitol and galactitol. The complete composition of the sugars and sugar alcohols measured in the phloem sap of *B. napus* is presented in Fig. 4 B and in the Table IV in the appendix. Extreme disproportions could be observed between the levels of two major compounds from this group (glucose-6-phosphate and myo-inositol), and the remaining sugars being far less abundant as presented in Fig. 4 B.

The third group of identified substances, the set of organic and inorganic acids, contained 21 metabolites. Within this group the most abundant were pyroglutamate, phosphate, citrate and D-isoascorbic acid. The distribution of the measured acids is

presented in Fig. 4 C. Many metabolites within this group were represented at very low levels with significant differences between the most and less abundant acids.

Many amino acids in the stem were detected at similar high levels (normalised values between 40000 and 60000) with oxoproline and proline being the most abundant (Fig. 5 A). Significant numbers of metabolites, however, were hardly detectable (i.e. uracil, S-met-cysteine, β-alanine, tryptophan). Similar to phloem sap, stem extracts contained mainly proteogenic amino acids, with glutamine being highly abundant. Within the group of measured sugars (excluding glucose, sucrose and fructose for the reason explained above), myoinositol was the most abundant sugar alcohol in the stem. The level of detected myoinositol was approximately 50 times higher than that of the next most concentrated sugar – mannitol. All remaining sugars were detected at similar levels. Glucose, fructose and sucrose concentrations were additionally measured with enzyme assays, showing glucose as the most abundant of these three sugars (Fig. 3).

From the group of inorganic and organic acids malate, ascorbate and citrate (Fig. 5 C) were most abundant. Many acids remained hardly detectable in the stem e.g. maleate that was detected in the phloem sap at relative high levels.

Measured phosphate level (plotted on the same diagram as inorganic and organic acids) was almost twice higher than most abundant acids.

The list of metabolites together with the measured values in the stem of *B. napus* is accessible in the appendix, Table IV.

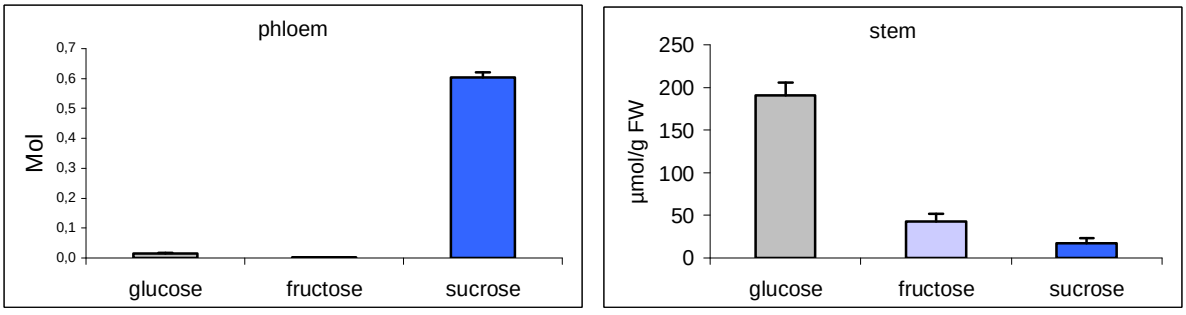

Fig. 3 Concentrations of glucose, fructose and sucrose in phloem sap and stem extracts of *B. napus*. The most abundant sugar in phloem sap of rape was sucrose (0.6 Mol) while fructose was at the detection limit. In the stem, glucose concentration (represented in µmol/g fresh weight) was the highest and fructose and sucrose were much less concentrated.

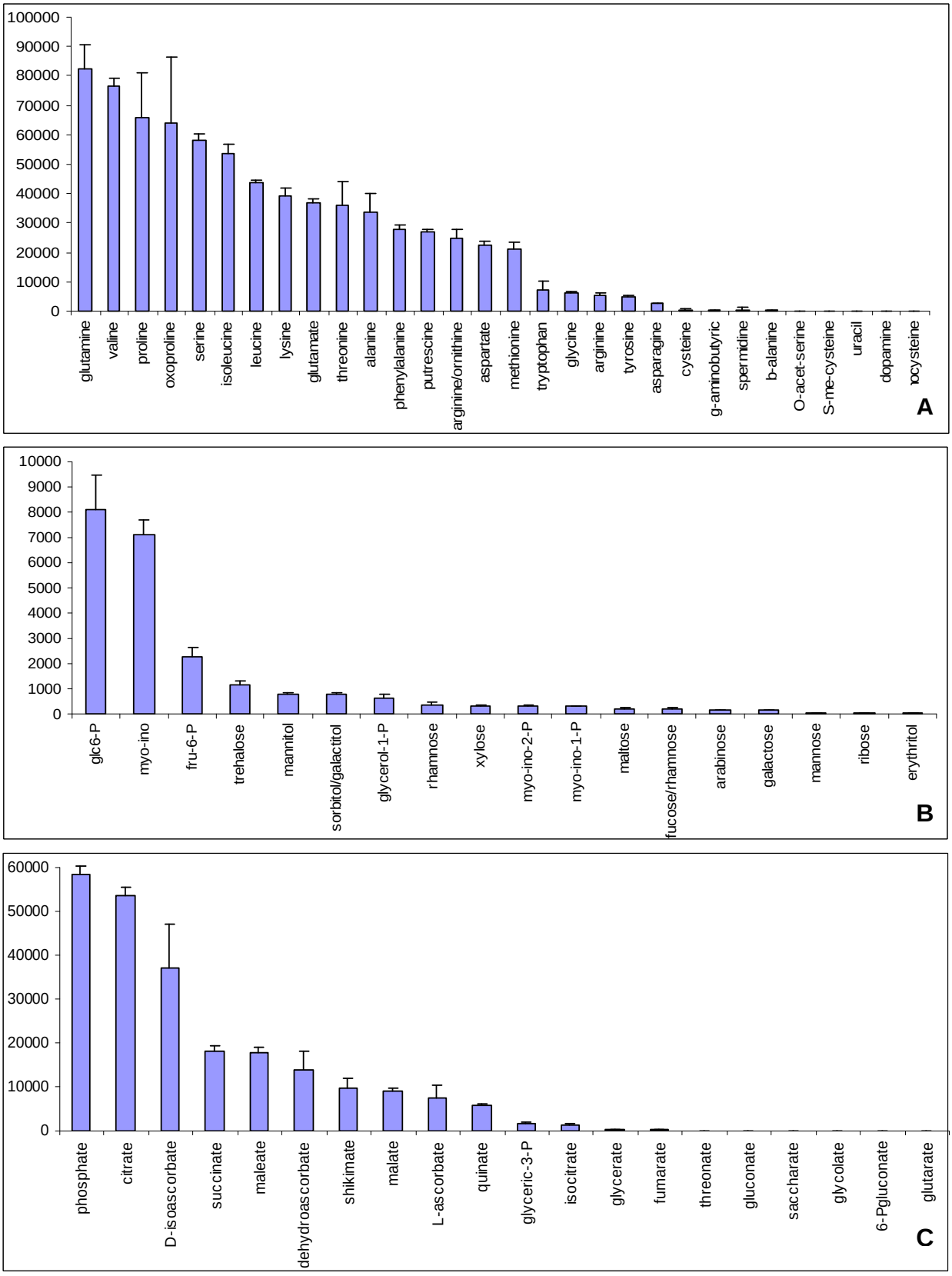

Fig. 4 Distribution of metabolites in the phloem sap of *B. napus* measured by GC-MS. Relative levels of amino acids and amines (A), sugars and sugar alcohols (B) and organic and inorganic acids (C) were normalised to the complete peak area of all identified metabolites within one sample.

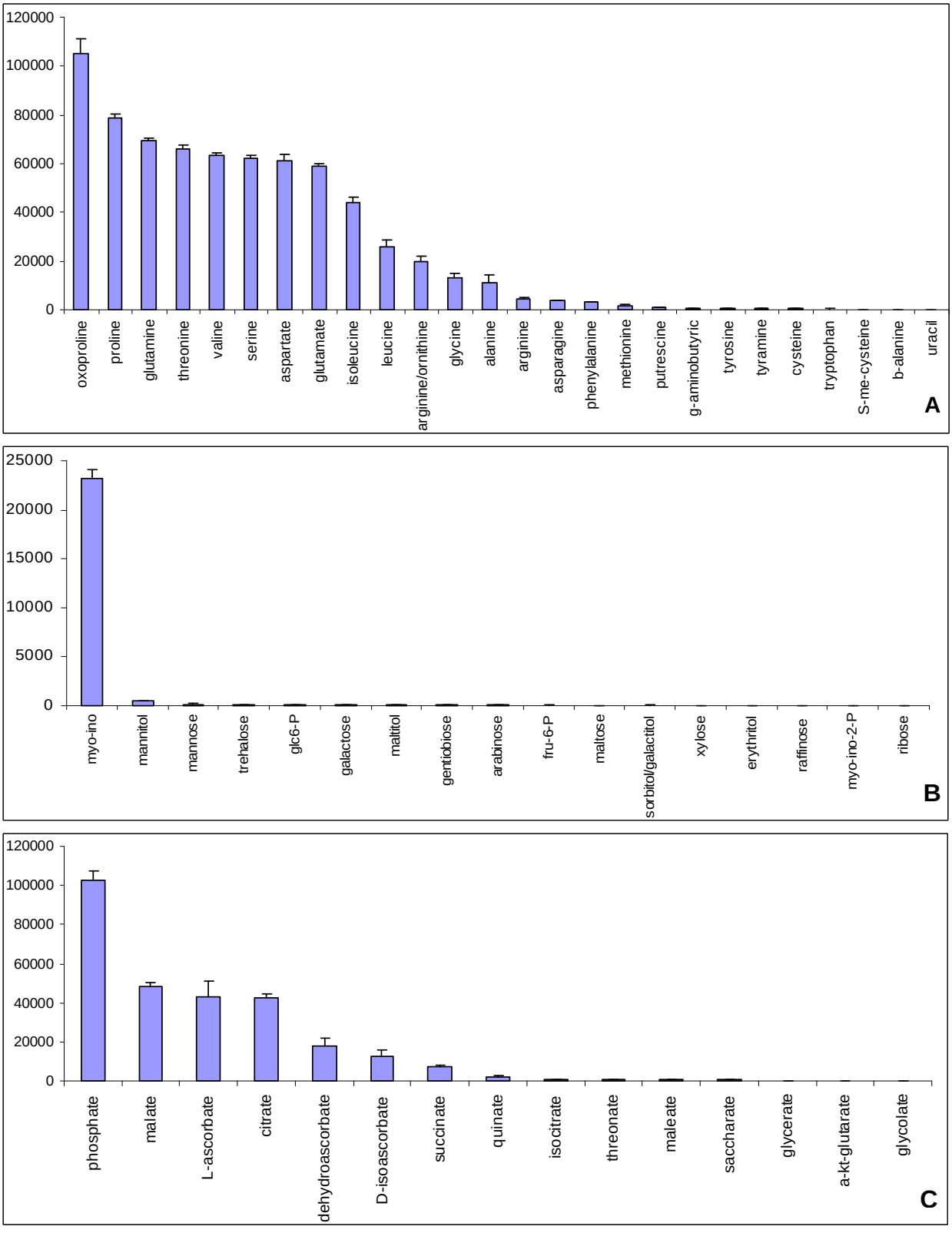

Fig. 5 Distribution of metabolites in the stem of *B. napus* measured by GC-MS. Relative levels of amino acids (A), sugars (B) and organic and inorganic acids (C) were normalised to the complete peak area of all identified metabolites within one sample.

3.1.3.2 Comparisons of metabolite compositions in phloem sap and stem tissue

To verify that the metabolite composition in the phloem differs from complete stem metabolite content, the results from GC-MS metabolite profiling of phloem and stem samples were compared by calculating phloem vs. stem ratios. The results are presented in Fig. 7.

Generally, higher levels of amino acids and sugars were detected in the phloem sap (Fig. 7 A, B), while organic and inorganic acids were slightly higher in the stem (Fig.7 C). Several metabolites like tyramine, gentobiose, maltitol, raffinose and α-keto glutaric acid appeared exclusively in the stem tissue and some were detected exclusively in phloem sap (lysine, o-acetylo serine, glycerol-1-P, myoinositol-1-P, rhamnose, shikimic and fumaric acid, spermidine) (data available in the appendix, Table IV). Many of the amino acids (threonine, glutamic acid, asaparagine, S-met-cysteine, proline, serine, glutamine, valine, isoleucine, arginine, leucine) were present at similar levels (less than one fold change) in both tissues. From the amino acids detected in different amounts, only aspartic acid and glycine were more than one fold higher in stem than in phloem sap, while many were measured at higher levels in phloem sap than in the stem (cysteine, alanine, b-alanine, phenylalanine, tyrosine, methionine, putrescin, tryptophan).

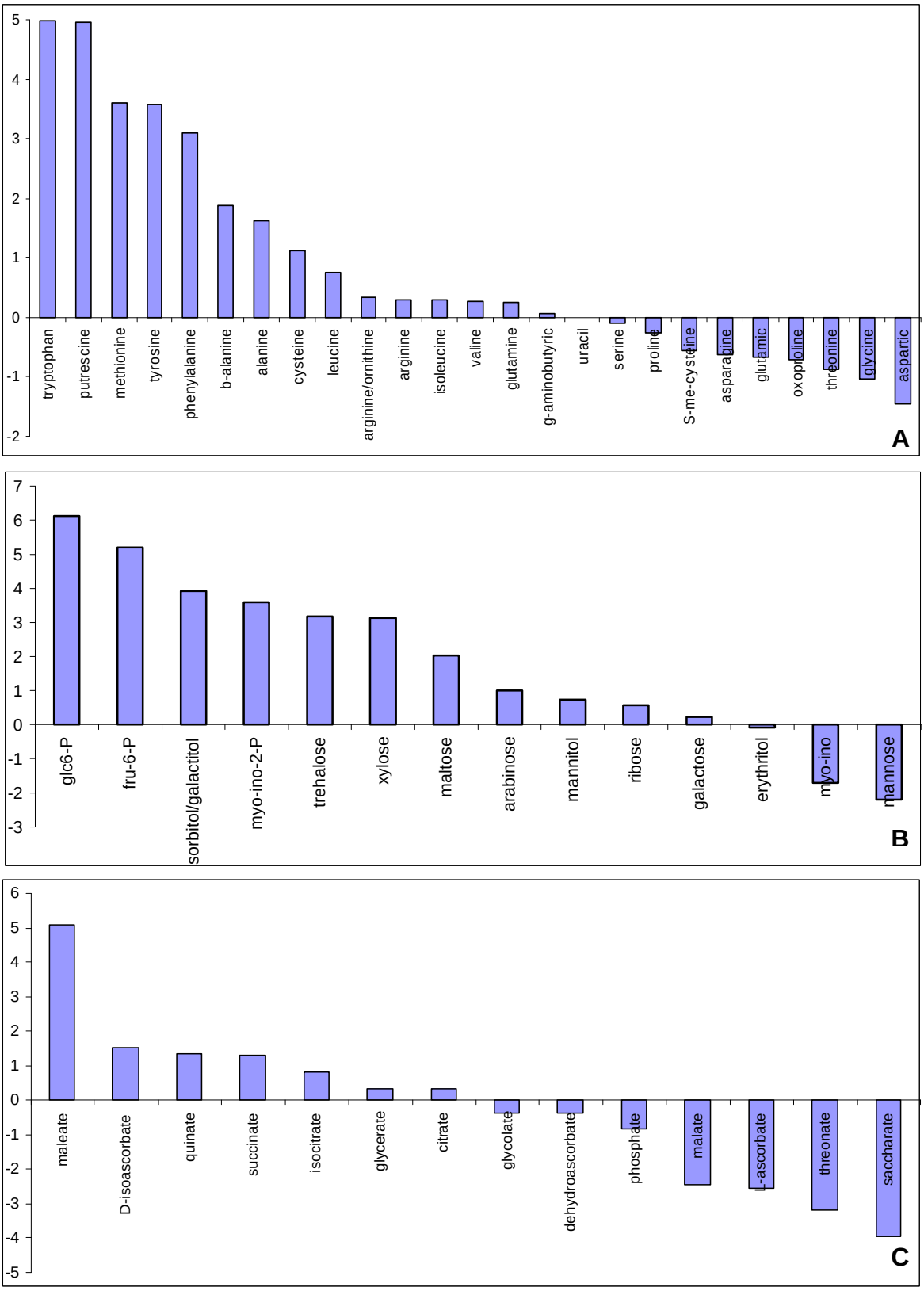

Fig. 5 Ratios (log4 values) of the relative metabolite content measured by GC-MS in phloem sap compared to stem extracts. Ratios of amino acids (A), sugars (B) and organic and inorganic acids (C) calculated from values normalised to the complete peak area of all identified metabolites within one sample.

3.2 Identification of phloem proteins using expression libraries and anti-phloem protein specific antibodies

3.2.1 Anti-phloem protein specific antibodies

Many proteins detected in the phloem so far remained unidentified and an immunological approach employing antibodies constitutes a powerful tool, complementary to 2 DE MS/MS method, for protein identification. Antibodies, due to their sensitivity and specificity, were used in this study to discover novel phloem proteins. To achieve this, antisera against complete proteins from phloem sap of *C. sativus*, *C. maxima* and *B. napus* were raised in rabbits. Phloem sap was simultaneously collected from several plants of one species to obtain sufficient amounts (>1 mg) of protein for immunisations. The produced antisera were tested for cross reactivity with leaf extract from the respective plant and phloem exudates from every examined species. Immunoblot analysis showed that the antisera reacted exclusively with the proteins they were raised against. Interestingly, no cross reactivity was observed between anti *C. sativus* phloem protein antiserum and phloem proteins from the related plant *C. maxima* (Fig. 8). In addition, no signal was observed when this antiserum was tested with leaf extracts of *C. sativus* or with phloem proteins from *B. napus*. Antibodies directed against phloem proteins of *C. maxima*, reacting with *C. maxima* phloem proteins but not with *C. maxima* leaf extract, *C. sativus* or *B. napus* phloem proteins, showed a similar behaviour (Fig. 8). Antiserum against *B. napus* phloem proteins similarly did not cross react with *C. sativus* or with *C. maxima* phloem proteins (Fig. 8). No cross reactivity of all tested anti phloem antisera with any proteins from different tissues tested or with phloem proteins from other species demonstrated that these antisera were very specific.

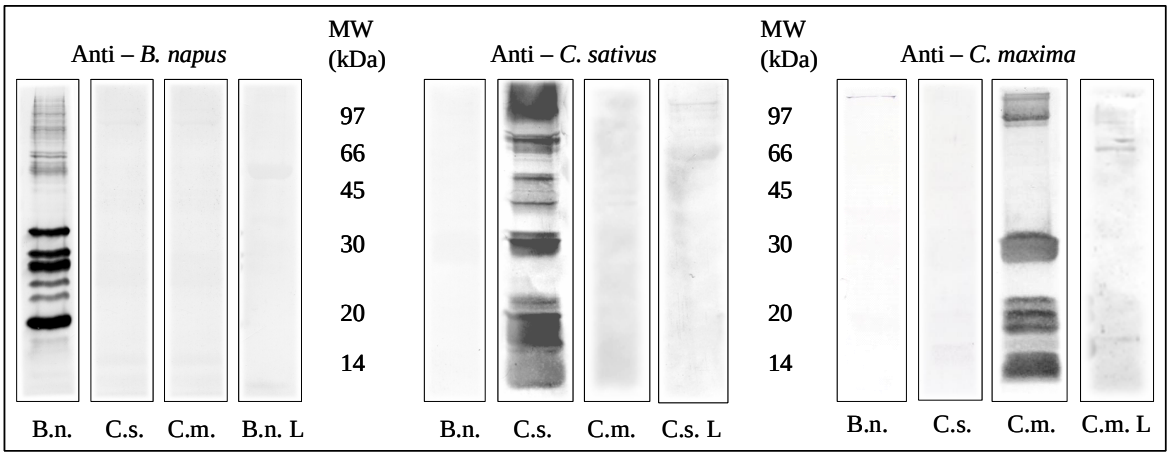

Fig. 6 Reactivity of antibodies raised against phloem sap proteins from various species. Phloem sap (10 µg protein per lane) from *B. napus* (B.n.) *C. sativus* (C.s.) *C. maxima* (C.m.) and leaf crude extracts from the corresponding species (L) were separated on 1DE, blotted and probed with antibodies. Pre-immune sera did not cross-react with phloem proteins (data not shown). Probed antisera reacted exclusively with the phloem proteins they were produced against.

3.2.1.1 Immunolocalisation

To further confirm the phloem protein specificity of the produced antisera, immunolocalisation studies were performed. Stem tissue printing and whole leaf electroblotting techniques were employed for these experiments. Young stems of *C. sativus* (Fig. 9 a), *B. napus* (Fig. 9 b) and *C. maxima* (data not shown) were used for tissue prints on nitrocellulose membranes. Membranes probed with the corresponding antisera all showed the same clear phloem-specific pattern. The labelling of vascular bundles of *B. napus* and additional extrafascicular phloem vasculature in *C. sativus* can be distinguished from the complete stem pictures (Fig. 9). No cross reactivity with xylem or phloem parenchyma could be observed. Electroblots of complete leaves of *C. sativus* (Fig. 9 c) and *C. maxima* (not presented) confirmed that the tested antisera localised exclusively to the phloem. Amido-black total protein staining of electroblotted leaf proteins was performed for comparison.

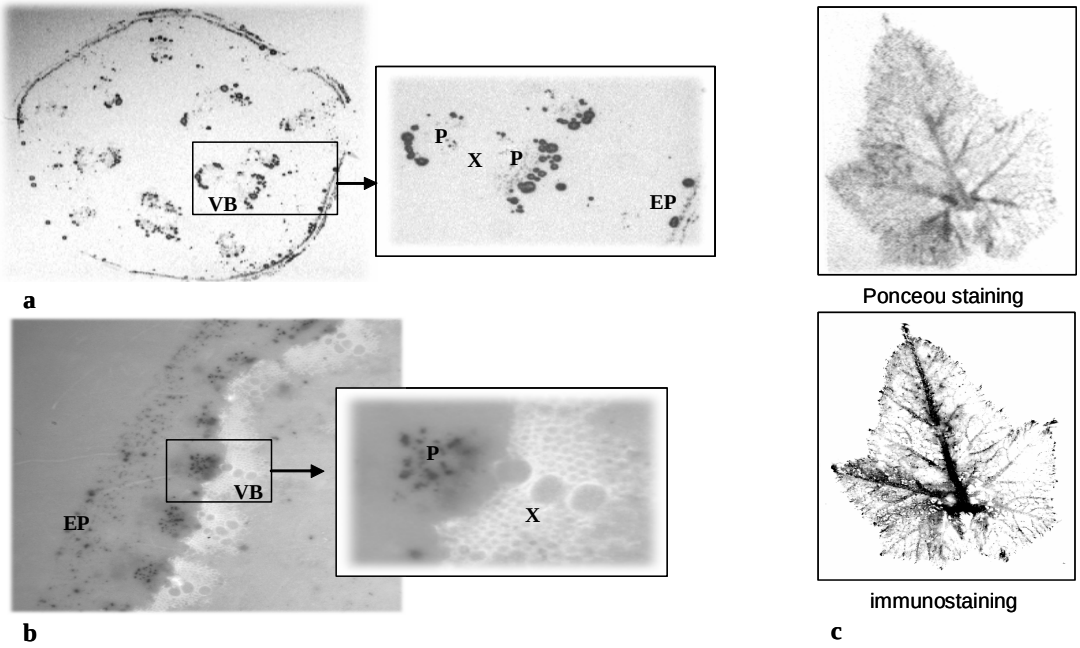

Fig. 7 Immunolocalisation of anti-phloem protein antibodies on stem tissue prints and leaf electroblots. Transversely cut stems of *C. maxima* (a) and *B. napus* (b) was pressed several times on nitrocellulose membranes. These tissue prints were incubated with antibodies raised against phloem proteins from *C. maxima* (a) and *B.napus* (b), respectively. In both cases the antibodies localised specifically to the phloem. (c) Electroblots of young, dewaxed leaves of *C. maxima* were first stained for proteins with Ponceau (upper picture) followed by immunostaining using anti- *C.maxima* phloem protein antiserum (bottom) which localised to vascular tissue. VB –vascular bundle, P –phloem, X –xylem, EP – extrafaccicular phloem;

3.2.1.2 Staining for posttranslational modifications

It was possible that the unusually specific behaviour of the anisera might be caused by posttranslational modifications of the phloem proteins. It was previously described (Cronshaw and Sabnis, 1990) that some phloem proteins can undergo posttranslational modifications like glycosylations. The most common modifications of eukaryotic proteins are glycosylations and phosphorylations. Such modification sites might serve as highly antigenic epitopes during antibody production and could finally lead to the formation of antisera that recognise mainly modifications. To exclude the possibility that the observed specificity was due to a common posttranslational protein modification, glycosylated and phosphorylated phloem proteins were detected on SDS-PAGE gels and their patterns on the gels were compared to the antibody marked phloem proteins (Fig. 10 a, b). 1 DE gels were used for *C. maxima* and *C. sativus* protein analysis. Many glycosylated and phosphorylated proteins were detected in phloem sap of *C. maxima* and *C. sativus*. However, the patterns of

visualised modified proteins differed from that of antibody-marked polypeptides. This indicates that the tested antisera were produced not exclusively against glycosylation or phosphorylation sites on phloem proteins of cucumber and pumpkin. Similar results were obtained on rape phloem proteins (Fig. 10 c). Due to the high complexity of proteins in the phloem sap of rape, high-resolution 2 DE was performed in order to better separate proteins. Subsequent staining for glycosylated proteins displayed only one weak signal indicating that there were no abundant glycosylated proteins in the phloem of *B. napus* (data not shown).

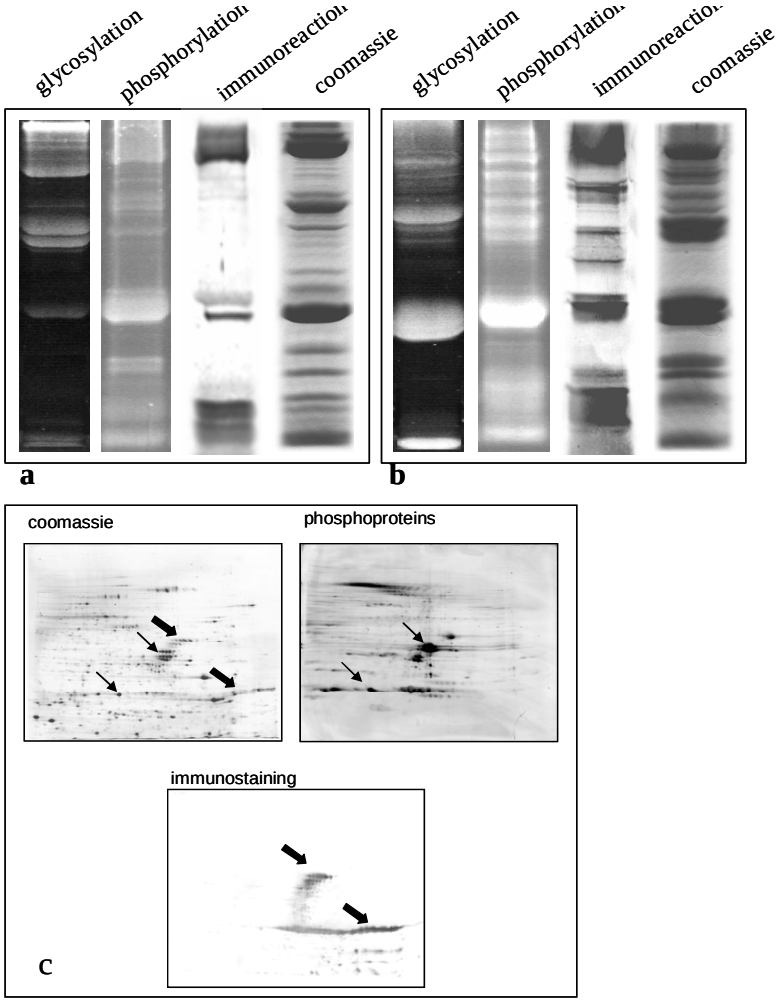

Fig. 8 Detection of post-translational modifications on phloem sap proteins. *Cucumis sativus* (a), *Cucurbita maxima* (b) and *Brassica napus* (c) phloem sap proteins (10 µg per lane) were separated by 1 DE (a,b) or 2 DE (c) SDS-PAGE gel electrophoresis. Proteins were stained in gel with colloidal Coomassie or blotted to membranes and used for western blotting with antisera (anti-phloem protein of *C. sativus* (a), *C. maxima* (b), *B. napus* (c), antiserum) or submitted to a detection of glycosylation and phosphorylation sites on proteins. While phosphorylated proteins were present in all three species examined, no glycosylated proteins were detected in phloem sap of *B. napus.*

Several proteins appeared to be phosphorylated but again the pattern of these modified proteins was different from that of antibody-stained proteins (Fig. 10). Thus, the examined common posttranslational modifications did not seem to cause the observed high antibody specificity.

3.2.1.3 Attempts to determine the specific epitopes

To test whether the anti phloem protein antibodies were produced mainly against one specific epitope, affinity purification of the rape antiserum was performed. Affinity purified antibodies were then used in western blots with phloem proteins (Fig. 11). For this experiment, phloem proteins were separated on 1 DE, blotted onto a nitrocellulose membrane, and stained for complete protein with amido black. Three major bands, that were marked by the rape phloem specific antiserum, assigned as sample 1 (protein ~31 kDa) sample 2 (~28 kDa) and sample 3 (~25 kDa), were cut out from the blot (Fig. 11 A) and separately incubated with complete antiserum to isolate the individual antibodies binding to these proteins. Antibodies bound to the proteins from sample 1, 2 and 3 were subsequently eluted from the membrane and tested on western blots with complete phloem proteins (Fig. 11B).

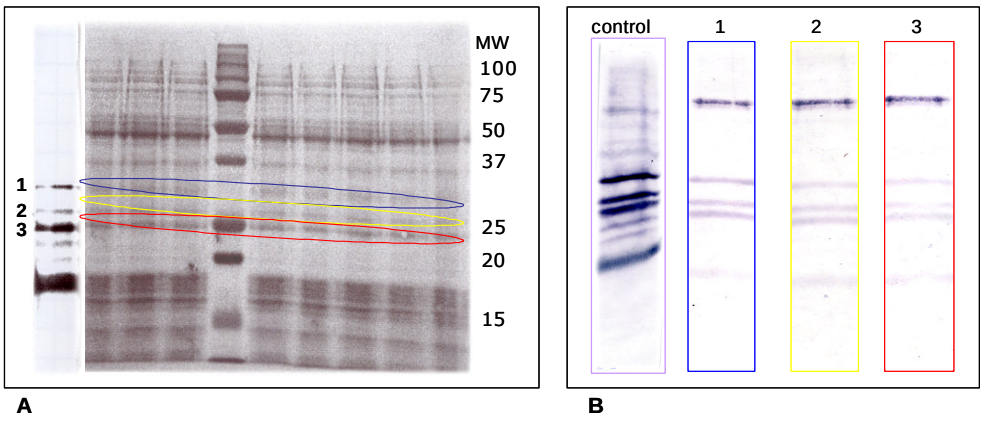

Fig. 9 Affinity purification of selected antibodies. Phloem proteins from *B. napus* were separated by one-dimensional SDS-PAGE gel electrophoresis, transferred onto nitrocellulose membranes and stained for proteins with amido black (A). One lane was cut out from the blot and incubated with anti-rape phloem protein antiserum to visualise the major protein bands reacting with the antibodies. From the nitrocellulose three different major protein bands were cut out and separately incubated with anti-phloem protein antiserum. Antibodies bound to each protein band were subsequently eluted from the nitrocellulose and their specificity was tested on the western blots with complete phloem sap proteins from rape (B). Antibodies that were separately purified with three distinct protein bands seem to react with the same epitope(s) because they show a similar pattern to the control reaction where complete antiserum was used.

Antibodies eluted from each sample seemed to recognise the same proteins within phloem sap. Therefore, it is likely that at least these three phloem proteins share a similar, highly antigenic epitope responsible for the reaction with the corresponding antiserum.

To reveal the nature of this epitope, phloem proteins that reacted with antiserum were purified by immunoprecipitation and analysed on 1 DE. Searches for the specific amino acid sequence that could form an epitope could be performed on discovered partial amino acid sequences of the purified proteins. Additionally, purified proteins could be analysed to verify the presence of possible molecules of unknown nature that can be bound to the peptide forming an epitope. The second approach would be investigated by MALDI-TOF method after complete protein digestion. For immunoprecipitation, IgGs from anti-rape phloem protein antiserum was bound to protein-A coupled ferrule beads and incubated with antigen- rape phloem proteins (0,6-0,8 mg of protein) (Fig. 12). Subsequent elution of antigen resulted in purified phloem proteins that specifically bind to the antibodies. These purified proteins were then separated on 1 DE and detected by protein staining or analysed on western blots for a control. To visualise purified proteins on the gel the very sensitive silver staining method was used. Control western blot analysis confirmed that the purified proteins do react with the tested antibodies (Fig. 12). However, the amount of eluted proteins was very low and did not allow the use of a staining method compatible with ESI-QTOF MS/MS that was planned for subsequent determination of partial amino acid sequences from these proteins. The very low amounts of purified protein hampered also MALDI-TOF analysis to search for possible specific molecules potentially bound to the proteins forming an epitope.

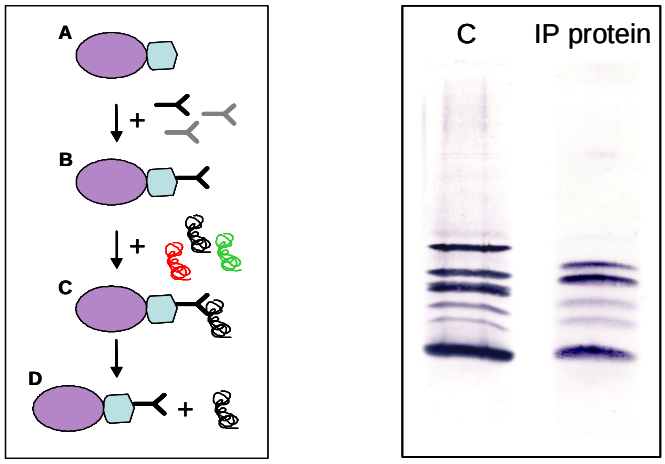

Fig. 10 Immnunoprecipitation of phloem proteins that bind to anti-rape phloem protein antibodies. Scheme of the immunoprecipitation procedure (left panel): A- Ferrule beads coupled with protein A are incubated with antibody containing sample (anti-phloem protein specific antiserum); B- IgGs from the antiserum bind to protein A, this complex is subsequently incubated with antigen (phloem proteins) and specific antigens bind to the corresponding IgGs -C. Proteins can be eluted from the complex -D. Western blot analysis of immunoprecipitated proteins from phloem sap of *B. napus* (right panel) C- Control sample of total phloem sap proteins and IP- immunoprecipitated proteins were incubated with anti-phloem protein specific antiserum. On protein gels, the IP proteins could only be detected as weak signals after silver staining (results not shown).

3.2.2 Construction of expression libraries

To investigate the structure and function of phloem sap-specific proteins it is necessary to obtain full-length DNA sequences of their coding genes. For this purpose, three independent phage cDNA expression libraries were constructed from total RNA of *C. maxima*, *C. sativus* and *B. napus*. The libraries were subsequently screened with the corresponding anti phloem protein-specific antisera and putative clones of phloem proteins were selected.

The libraries were constructed in a lambda phage vector that enables protein expression in prokaryotic *E. coli* cells. Cucumber and pumpkin lambda Zap libraries were created from existing libraries by sub cloning the inserts from the vector pCMV-Sport6.1 into the lambda ZapExpress vector. The originally constructed pumpkin library, before amplification, contained 7.8×10^6 pfu (plaque forming units) in a total volume of 500µl phage suspension. The cucumber library was formed of 5×10^6 pfu in a total volume of 500 µl. About 80% of clones from the pumpkin library and about 70% from the cucumber library appeared to contain inserts, as tested by a blue/white colony screen. The average insert sizes were checked by isolating inserts from

several randomly chosen plaques from both libraries and were in the range of 0.7-2.5 kb (Fig. 13 A, B).

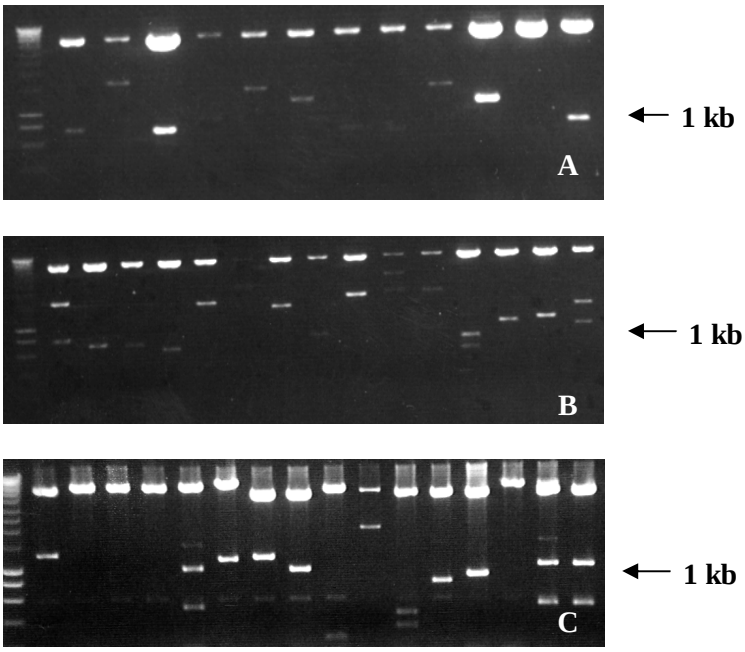

Fig. 11 Quality test of the expression libraries. Of each expression library (A- cucumber, B- pumpkin, C- rape) the insert sizes of randomly chosen clones were tested by cutting out the inserts from the vector and estimating their sizes after agarose gel electrophoresis. For all libraries the average insert size was approximately 1kb.

The rape expression library was constructed from 5 µg of total mRNA from *B. napus*. The originally produced library contained 5×10^5 plaque forming units in a total volume of 1000 µl. About 80% of the clones contained inserts and their average size was approximately 1kb (Fig. 13 C).

A subsequent amplification of the created libraries was performed to increase the number of clones and therefore simplify the library screening procedure.

Considering the expected relatively small representation of phloem specific transcripts within the total mRNA used for library construction, cucumber and pumpkin libraries were tested for the presence of CSF-2 cDNA. CSF-2 has been described as a fruit ripening and wound induced protein (Suyama et al., 1999) and the protein was previously identified in phloem sap of cucumber and pumpkin (Haebel and Kehr, 2001; Walz et al., 2004). With gene specific primers for CSF-2 a

radiolabelled cDNA probe was constructed and used for screening the libraries. After three rounds of screening, single positive clones were isolated and sequenced. The resulting sequences were compared to CSF-2 genes present in the NCBI database. As presented in Fig. 14 (A), the full length clone isolated from the *C. sativus* library was identical to the sequence previously identified in cucumber, while the sequence obtained from the *C. maxima* library differed only in three amino acids (Fig. 14 B). This demonstrated that transcripts of phloem sap proteins are contained in the library and therefore a screening with the phloem-specific antisera was conducted.

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A.    MSLCGKLEKDVPIRASASKFHEMFHKKPHHICNCSTDKIHGVELQEGEWGQVGS  - cucumber CSF2
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::
      MSLCGKLEKDVPIRASASKFHEMFHKKPHHICNCSTDKIHGVELQEGEWGQVGS  - sequence from the cucumber library

IICWKYFHEGKHKIAKEIVEHVDEENNSITFKVIEGDLTEHYKDFRFTIKCIPKEKGSVI
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
IICWKYFHEGKHKIAKEIVEHVDEENNSITFKVIEGDLTEHYKDFRFTIKCIPKEKGSVI

HWVLEYEKLHDKIPDSHTLLQFCVDVSKDIDKQLSAN
::::::::::::::::::::::::::::::::::::
HWVLEYEKLHDKIPDSHTLLQFCVDVSKDIDKQLSAN


B.    MSLCGKLEKDVPIRASASKFHEMFHKKPHHICNCSTDKIHGVELQEGEWGQVGS  - cucumber CSF2
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::
      MSLCGKLEKDVPIRASASKFHEMFHKKPHHICNCSTDKIHGVELQEGEWGQVGS  - sequence from the pumpkin library

IICWKYFHEGKHKIAKEIVEHVDEENNSITFKVIEGDLTEHYKDFRFTIKCIPKEKGSVI
:::::::::::::::::::::::::::: :::::::::::::::::::::::::::::::
IICWKYFHEGKHKIAKEIVEHVDEENNSIPFKVIEGDLSEHYKDFRFTIKCIPKEKGSVI

HWVLEYEKLHDKIPDSHTLLQFCVDVSKDIDKQLSAN
:::::::::::::::::::::::::::::::::: ::
HWVLEYEKLHDKIPDSHTLLQFCVDVSKDIDKQLFAN
```

Fig. 12 Translated amino acid sequences of the CSF2 cDNA clones isolated from the cucumber (A) and pumpkin (B) libraries, respectively. Sequences are aligned to the cucumber CSF2 sequence present in the database (accession no. Q9SXL8) using the LALIGN software (http://www.ch.embnet.org/software/LALIGN_form.html).

3.2.3 Expression libraries screening

Approximately 50,000 clones from each library were screened using the anti-phloem protein antiserum from the corresponding species. cDNAs isolated from positive clones were sequenced and database searches were performed to identify these cDNAs. The identification rate of the *C. sativus* and *C. maxima* clones was limited by the relatively poor genome coverage of *Cucurbitaceae*. Three rounds of screening were indispensable for the selection of single positive clones. Screening of the *C. sativus* library resulted in approximately 40 positive clones and from *C. maxima* 18 positives could be isolated. The insert size estimation of the tested clones allowed the selection of putative full-length sequences. Inserts composed of less than 600 nucleotides were discriminated and larger fragments were assigned as putative full-length clones and selected for sequencing. From the *Cucumis sativus* expression library approximately 20 clones were sequenced, as were 12 from the *Cucurbita maxima* library. More than 60 positive clones were isolated from the *Brassica napus* expression library. However, the majority (about 60%) of clones were of the same size, about 650bp, and only a few of them were selected for sequencing. In total, 27 cDNA fragments were sequenced from the *B. napus* library. In order to identify the clones, sequences were used for homology searches in the NCBI database. Within the 27 sequenced clones from the *B. napus* library, 5 were identified (Table V in appendix). One of them, thioredoxin h, has already been described in the phloem of other species (Ishiwatari et al., 1995). Although many of the sequenced cDNAs from *C. sativus* showed no homology to database entries (see Table V in appendix), it was possible to identify ten clones and assign them to six independent proteins (several clones were homologous to the same gene). From the *C. maxima* library, from 11 identified clones, 4 independent proteins could be distinguished. Identified clones are listed in Table 1.

Two full length clones from the *C. sativus* library were identical to a 17 kDa phloem lectin (Table 1), one of the major phloem proteins from *Cucumis sativus* (Dinant et al., 2003). This finding indicates the phloem protein specificity of the antiserum used for screening.

Interestingly a clone similar to a 14-3-3 protein was found in both *B. napus* and *C. sativus* libraries.

Most of the identified proteins, including 14-3-3, dehydrin, and rubisco activase had never been described to be present in the phloem before.

Protein match	mRNA source	plant	accession	% identity	overlap nt/ total nt in mRNA
Dehydrin	*C. m.*	*C. paradisi*	AY160772	61,4	946/1020
LHS1	*C. m..*	*N. tabacum*	AF166277	76,2	480/480
14.3.3	*C. s.*	*V. faba*	AB050952.1	76,1	665/1102
17kDa phloem lectin	*C. s.*	*C. sativus*	AF520578	100	465/465
Rubisco activase 1	*C. s.*	*G. hirsutum*	AF329934	77,9	981/1540
Esterase	*C. s.*	*A. thaliana*	NM_129716	74,9	466/1197
Cytohrome b5 domain	*C. s.*	*A. thaliana*	NM_128052	76,8	272/522
Transcriptional -coactivator like	*C. m.*	*A. thaliana*	AY042850	74,6	461/724
Transducine family prot.	*C. m*	*A. thaliana*	NM_112175	70,8	1088/4510
14-3-3-like protein	*B.n.*	*A. thaliana*	U02565	83,1	635/835
Thioredoxin-h-1-like	*B.n.*	*B.napus*	X89759	92,5	545/664
RIO1 family protein	*B.n.*	*A. thaliana*	NM_128057	85,5	1614/1614
Plastocyanin	*B.n*	*A. thaliana*	NM_106259	81,6	534/771
Double-stranded DNA-binding family protein	*B.n*	*A. thaliana*	NM_179397	86,1	660/812

Table 1 Significant matches of the cDNA sequences obtained from expression library screening; *C. s. Cucumis sativus, C. m. Cucurbita maxima, B. n. Brassica napus.* The percent of identity were estimated using the LALIGN software (http://www.ch.embnet.org/software/LALIGN_form.html).

3.3 Identification of phloem proteins using 2 DE followed by mass spectrometric analysis

3.3.1 Gel electrophoresis

An alternative method for phloem protein identification used in this work was the separation of polypeptides on high resolution 2 DE followed by tandem mass spectrometry of selected, proteolytically digested proteins. Analysis of the resulting partial amino acid sequences can lead to an identification of proteins through similarity searches against the sequences present in the NCBI database. Many proteins from *C. sativus* and *C. maxima* phloem have already been identified using such a mass spectrometric approach (Kehr et al., 1999; Walz et al., 2002; Walz et al., 2004). In this work, the analysis of selected proteins from phloem sap of *B. napus* was approached. 200 µl of phloem sap, corresponding to 160 µg of protein was used for over night precipitation in acetone/methanol/DTT solution. Proteins were next resolved and applied on the first dimension isoelectrofocusing gel where they are separated according to their electrical charge. The second dimension - reducing SDS-PAGE electrophoresis - distinguishes proteins by their masses. The proteins

were separated in a pH range of 4-10 and a molecular weight range of 5-150 kDa. After the separation, proteins can be visualised by several staining techniques. The very sensitive, commercially available silver staining method (detection limit of approx. 0.5 ng protein) is known to be incompatible with mass spectrometric analysis. Therefore, another method for protein staining, coomassie-blue (detection limit of approx. 50 ng protein) that has been proven to be compatible with mass spectrometric analyses was used. It was possible to visualise approx. 40 bands after 1 DE, resulting in more than 600 spots on 2 DE from rape phloem samples (Fig. 15). In comparison, silver staining of analytical gels allowed the detection of more than 1000 protein spots (data not presented).

3.3.2 *De-novo* sequencing of tryptically digested peptides using tandem mass spectrometry

For the identification of the separated proteins, partial sequences of trypsin digested peptides were analysed by Q-TOF (quadrupole-time-of-flight) tandem MS (mass spectrometry) fragmentation. For the MS analysis, coomassie-stained spots were selected, manually excised and digested with trypsin. Tryptic peptides were then introduced into a Q-TOF instrument, certain peptides isolated by quadrupole MS, fragmented, and subsequently the resulting fragments were detected by TOF MS. Sequence information can be derived from these fragmentation spectra. In the present study, as many peptides from each protein as possible were selected and submitted to collision induced fragmentation. For this purpose, fragmentation spectra were software processed and the amino acid sequence was determined manually using special software (Micromass).

These partial sequences were subsequently used for database similarity searches.

3.3.3 Protein identification

The results presented here are part of a larger project that is running in the group of J. Kehr. In the context of this project, all together 135 soluble proteins from rape phloem sap were identified from 2 DE by their high sequence similarity to database entries using either a matrix-assisted laser desorption ionisation coupled with

quadrupole time-of-flight MS (MALDI-Q-TOF) and/or a Q-TOF MS approach (Giavalisco et al., 2005).

Within this thesis, several of these proteins were identified from 2 DE gels after proteolytic digestion and partial sequence determination using Q-TOF tandem MS fragmentation.

A protein was regarded as identified if at least eight amino acids from a determined partial sequence showed 100% homology to a database entry or if at least two different sequences from one digested protein spot showed more than 70% similarity to the same database entry.

The proteins identified in this work are marked in Fig. 15 with numbers, and are listed in the Table III in the appendix. Some of the proteins that are of importance for this thesis and were identified within the bigger project are marked with asterisks in Fig. 15.

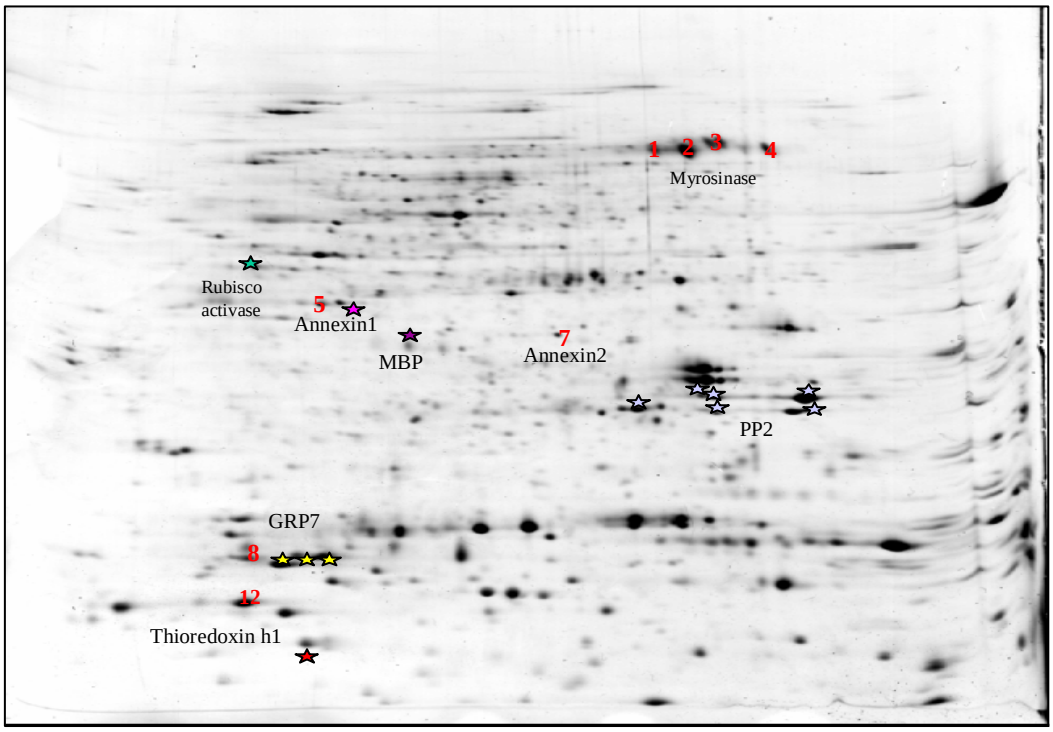


Fig. 13 Proteins identified by the 2DE-MS approach. Spots marked with numbers were identified in this work by ESI-MS/MS. Spots of importance for the thesis and identified within a larger project are marked by asterisks. From four spots identified as myrosinase (No 1-4), 9 partial amino acid sequences were obtained with MS. Two spots (No 5 and marked with asterisk) were identified as annexin 1 (10 partial sequences) and one (No 7) as annexin 2 (3 partial sequences). Four spots (No 8 and marked with asterisk) showed very high homology to glycine-rich RNA-binding protein 7 (4 partial sequences from spot 8), three of these spots were identified by MALDI-MS. From spot No 12 three partial sequences that showed high homology to thioredoxin h 1 were obtained and the neighbouring spot marked with asterisk was also identified as thioredoxin h 1. Partial sequences used for identifications are accessible as supplemental data.

One of the highly abundant protein classes identified on the 2 DE gels were myrosinases (marked with numbers 1-4 in Fig. 15). Additionally, myrosinase binding proteins were identified (marked with asterisk) that, together with myrosinases, are components of the myrosinase defence system against microorganisms and insects (Lenman et al., 1990; Rask et al., 2000). As can be seen in Figure 15, the myrosinase proteins seem to be highly modified, indicated by a number of distinct protein spots they were identified in.

Additional possible defence and wound-induced proteins found in exudates are phloem lectins similar to PP2 cucurbit phloem lectin (Fig. 15).

The identified annexin 1 and annexin 2 (spot 5, 6 and 7 respectively) are Ca^{2+} binding proteins. Another abundant class of phloem proteins detected in this study, the glycine-rich RNA binding proteins (spot 8 and neighbouring spots marked with yellow asterisk) have the properties to bind RNA.

One of the most abundant phloem proteins in rice is thioredoxin h (Ishiwatari et al., 1995). Its analogue was also detected in the phloem of *B. napus* in the present work with both, the 2 DE MS/MS approach (spot 9 and neighbouring spot marked with asterisk) and the expression library screening studies.

3.4 Confirmation of the phloem localisation of identified proteins

To proof the phloem localisation of proteins identified from the expression libraries and/or by partial sequences obtained from mass spectrometric analyses, an immunological approach was employed. Available antibodies directed against specific proteins were used to probe phloem exudates for the presence of these polypeptides.

As described earlier, the sequence of a dehydrin was identified in the library of *C. maxima*. Polyclonal peptide antibodies directed against dehydrin (Close et al., 1993) were used to probe for the presence of similar proteins within the phloem sap of the investigated species. Dehydrin appeared to be present in the exudates from all tested plants with the highest abundance in *B. napus* (Fig. 16). An additional abundant cross-reactive band at 28 kDa in *C. maxima* phloem was detected, while several bands appeared in the phloem of *B. napus*. Dehydrin was not detected in leaf extracts of the examined plants. To verify these results, western blots from 2 DE gels

were performed and the previously used anti dehydrin antibodies were employed. As shown in Fig. 17, weak signals were visible on the 2 DE blot.

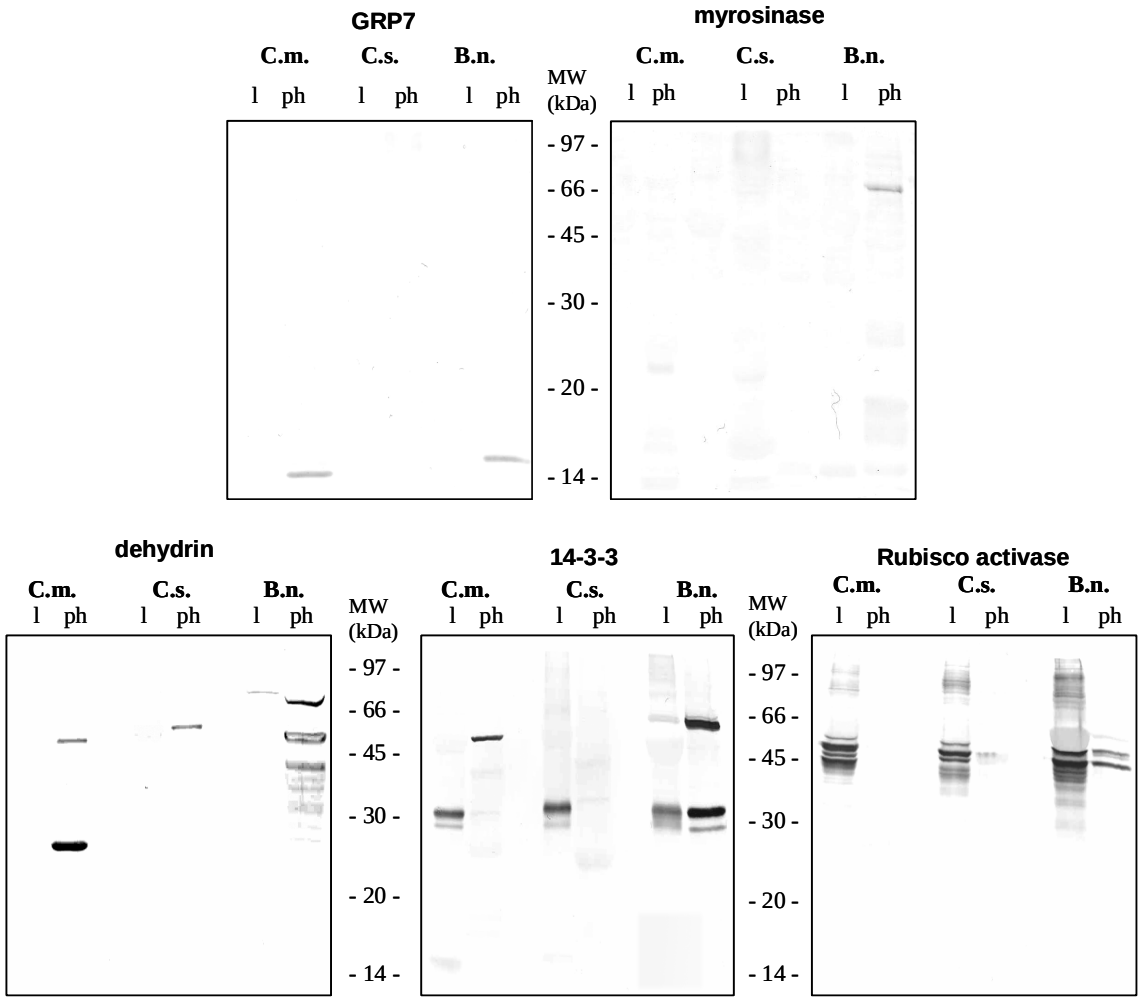


Fig. 14 Verification of the presence of dehydrin, 14-3-3 protein, rubisco activase, GRP7 (glycine-rich RNA-binding protein 7) and myrosinase in the phloem sap of *Brassica napus* (B.n.), *Cucumis sativus* (C.s.) and *Cucurbita maxima* (C.m.). Phloem saps (ph) and crude leaf extracts (l) (5 µg protein per lane) were separated by SDS-PAGE gel electrophoresis, and subjected to Western blot analysis with anti-dehydrin, anti-14-3-3, anti-rubisco activase, anti- GRP7 and anti-myrosinase antibodies. Rainbow 756 MW marker was used for estimation of protein molecular masses.

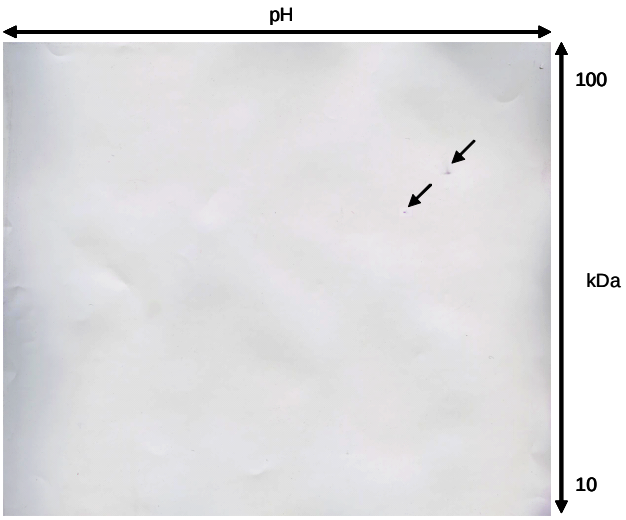


Fig. 15 Detection of dehydrins in phloem sap of rape by western blotting from 2 DE. About 80 µg of phloem sap proteins were separated by 2 DE and blotted to the membrane. Anti-dehydrin primary antibodies and AP-conjugated anti-rabbit IgG secondary antibodies were used for the detection. Two signals appeared identifying dehydrin.

Also sequences with high similarity to 14-3-3 proteins were found in both the *C. sativus* and the *B. napus* libraries. The presence of 14-3-3 proteins in the phloem was verified by western blot analysis using polyclonal antibodies against a 14-3-3 protein (Wilczynski et al., 1998) (Fig. 16). Anti 14-3-3 antibodies recognised two polypeptides at approx. 30 kDa in leaf extracts of all tested species and phloem from *B. napus* and an additional 66 kDa protein in the phloem of *C. maxima* and *B. napus*. No signals were detected in the phloem of *C. sativus*.

Another protein, rubisco activase 1, was identified from the *C. sativus* library (Table 1). Western blot analyses using monospecific polyclonal antibodies directed against a recombinant rubisco activase from tobacco (Feller et al., 1998) indicated that the phloem sap of *B. napus* contained higher levels of detectable activase then *C. sativus*. Interestingly, no cross-reactivity was observed with phloem sap of *C. maxima*. As expected, high levels of several rubisco activase 1 isoforms were detected in protein extracts from leaves of all three species.

Myrosinases, abundant phloem polypeptides identified from rape 2 DE gels by partial sequences similarity (Fig. 15) are characteristic for the *Brassicaceae* family

(Andreasson et al., 2001). They were therefore not expected to occur in the phloem of cucurbits. Accordingly, monoclonal antibodies against rapeseed myrosinase detected myrosinase only in the SE exudates from rape, while no signals were observed in the phloem of cucurbits or in leaf extracts (Fig. 16). The detected band was in the size of approximately 75 kDa, which is consistent with our 2 DE analysis (Fig. 15) and previous studies (Rask et al., 2000).

In this study, another abundant class of phloem proteins identified by 2 DE/MS analysis were the glycine-rich RNA biding proteins (GRPs). Polyclonal antiserum was produced against a 14 amino acid peptide from the identified GRP 7 protein to verify its localisation in the phloem. The peptide chosen for immunisation, IDSKIINDRETGRS, lies outside the glycine-rich domain of the protein. Western blot analyses using the produced antibody showed the presence of GRP 7 protein in the phloem of rape and pumpkin (Fig. 16). No GRP 7 was detected in the phloem of cucumber or leaf extracts from all tested species. The produced antiserum seemed to be specific for *B. napus* GRP 7, showing no cross-reactivity with other glycine-rich proteins from leaf extracts.

The group of annexin proteins, detected on 2 DE gels of rape phloem and identified by mass spectrometry analysis, are of great interest because of their Ca^{2+} sensing properties and are candidates for Ca^{2+} dependent signalling events that were proposed to occur in the phloem (Knoblauch et al., 2001; van Bel and Gaupels, 2004). Polyclonal peptide antisera against annexin were used to detect these proteins in the phloem. Two faint bands in the size of annexin 1 from Arabidopsis (Clark et al., 2005), being in the range of 35-42 kDa were visible on the western blots in phloem sap from *B. napus* and in stem extract of *A. thaliana* (data not presented).

3.5 Functional analysis of some identified phloem sap proteins

Identification of the proteins by the cDNA or peptide sequence homology does not give any information about their functionality within the phloem. To unravel the biological function of some of the identified proteins preliminary studies on *Arabidopsis* T-DNA insertion mutants were performed. Additionally, proteins with Ca^{2+} binding properties were investigated.

3.5.1 Ca^{+2} binding proteins

A significant group of proteins that can function as calcium sensors was identified in the phloem sap of the tested species. The group comprised annexin 1 and 2, calmodulin and a C2 domain containing protein identified with the 2 DE/MS approach (Giavalisco et al., 2005). To test whether some of these proteins functionally bind calcium in the phloem, an experiment with immobilised Ca^{+2} ions was performed. 800 µl of phloem sap from rape were run through a metal chelate column with calcium ions immobilised to imidoacetic acid groups covalently bound to a membrane. Proteins bound to calcium were eluted with imidazole and EDTA containing buffer. As can be seen in Fig. 18, 1 DE analysis showed that it was possible to elute specific proteins from the column. The pattern of eluted proteins was different from the complete protein pattern (the flow-through fraction) or the pattern from the elution of remaining proteins using SDS- buffer (Fig. 18). Visible bands of possible Ca^{+2} binding proteins were cut out, digested with trypsin and introduced to mass spectrometric partial sequencing. Sequence analysis would allow the identification of the proteins and comparison with the results obtained by 2 DE/MS. The amount of eluted protein was however so small that the MS analysis did not result in good quality spectra enabling the determination of partial amino acid sequences.

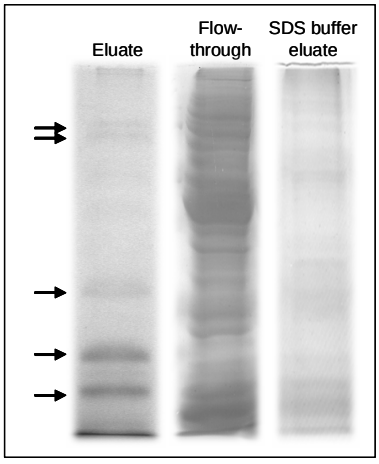


Fig. 16 Analysis of putative calcium binding proteins from phloem sap of *B. napus*. Purified phloem sap (400 µl) was loaded on a metal chelate column with boud calcium and Ca^{2+} binding proteins were eluted with buffer containing inositol and EDTA. Eluted proteins were separated by 1 DE and stained with coomassie. Visible bands (marked with arrows) were cut out and analysed by ESI-MS. Additionally proteins that remained on the column after selective elution were eluted with SDS-containing buffer and together with complete phloem proteins that did not bind to the column, the flow-through fraction, were separated by 1 DE. The patterns of the selectively eluted proteins differ from both control fractions suggesting a specific elution of only a few proteins that potentially bind calcium ions.

3.5.2 Analysis of T-DNA insertion knockout mutants of *Arabidopsis thaliana*

3.5.2.1 Selection of candidate proteins for functional analysis

The reverse genetic approach, namely the T-DNA insertion knockout mutant analysis, was chosen as a method for functional protein characterisation. The group of identified proteins from the phloem of *B. napus* offered the possibility to choose candidate proteins for these studies.

To first verify whether data from rape phloem sap can be transferred to Arabidopsis, the *B. napus* phloem protein-specific antiserum was tested against *A. thaliana* tissue prints. Stem tissue prints from *B. napus* and *A. thaliana* were incubated with this rape antiserum followed by the detection with secondary anti- rabbit IgG coupled to alkaline phosphatase. As can be observed in Fig. 19, the antibodies localised to the phloem region within vascular bundles of both species. The additional signals detected within parenchyma tissue (Fig. 19) seemed to occur exclusively in intercellular spaces and could therefore derive from phloem sap spread over the stem tissue during cutting. Accordingly, these signals became weaker with subsequent prints, while the signals from phloem remained unchanged (data not presented). These results provide evidence that at least some of the major proteins from rape phloem also occur in *Arabidopsis* vascular tissue.

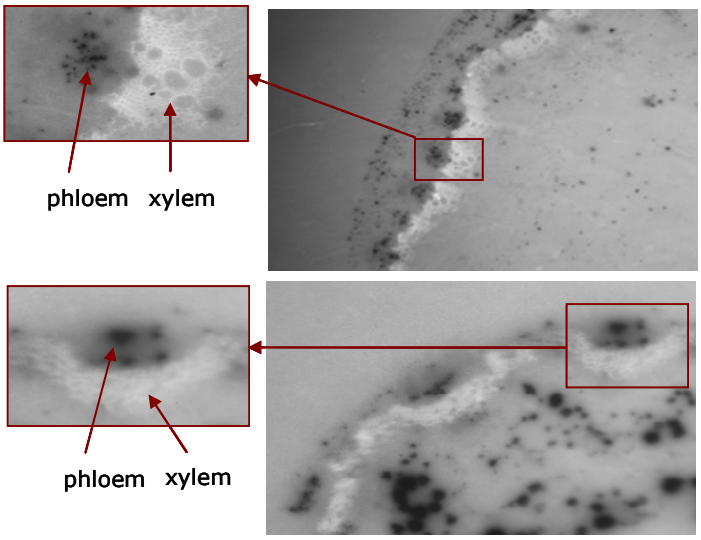


Fig. 17 *B. napus* and *A. thaliana* stem tissue prints on nitrocellulose incubated with anti *B. napus* phloem protein specific antiserum. The employed antibodies recognise proteins within vascular bundles of *B. napus* stem (upper panel) as well as from *A. thaliana* (lower panel). Additional signals observed in the middle parts of the stem were probably caused by phloem sap that spread over the section during sectioning of fresh, unfixed tissue.

Therefore the subset of phloem sap proteins identified by library screening and 2 DE MS/MS from rape formed the basis for the selection of candidate proteins for functional analysis. Several criteria were applied to select mutants from available *A. thaliana* T-DNA knockout lines. Firstly, only proteins that were abundant in phloem sap were considered in order to exclude possible contaminations. Secondly, phloem specificity of the genes of interest was inquired by sequence comparisons. Thus, only *A. thaliana* genes that showed a high homology to the partial amino acid sequences obtained form *B. napus* phloem protein analyses or/and genes with high similarity to the full length clone sequences obtained from the *B. napus* library screening were considered. For this purposes sequence comparisons between the most homologues *A. thaliana* and *B. napus* genes and partial amino acid sequences obtained from ESI-Q-TOF measurements were performed. Sequence comparisons for some of the proteins are presented in Figs. 20, 21 and 22. A further limiting step was the availability of specific knockout lines.

```
AtAnn1          MATLKVSDSV PAPSDDAEQL RTAFEGWGTN EDLIISILAH RSAEQRKVIR
AtAnn2          MASLKVPSNV PLPEDDAEQL HKAFSGWGTN EKLIISILAH RNAAQRSLIR
Ann1Phloem      ---------V PSPSEDAEQL ---------- ---------- ----------
Ann2Phloem      ---------- ---------- ---------- ---------- ----------

AtAnn1          QAYHETYGED LLKTLDKELS NDFERAILLW TLEPGERDAL LANEATKRWT
AtAnn2          SVYAATYNED LLKALDKELS SDFERAVMLW TLDPPERDAY LAKESTKMFT
Ann1Phloem      ---------- --KTLDKELT SDFER----- -------DAL LANEATKR--
Ann2Phloem      SAYAAAYNED LLKALDKELS SDFER----- ---------- ----------

AtAnn1          SSNQVLMEVA CTRTSTQLLH ARQAYHARYK KSLEEDVAHH TTGDFRKLLV
AtAnn2          KNNWVLVEIA CTRPALELIK VKQAYQARYK KSIEEDVAQH TSGDLRKLLL
Ann1Phloem      ---------- ---------- ---------- -------AHH TTGDFR----
Ann2Phloem      ---------- ---------- ---------- -SIEEDVAQH TSGDLR----

AtAnn1          SLVTSYRYEG DEVNMTLAKQ EAKLVHEKIK DKHYNDEDVI RILSTRSKAQ
AtAnn2          PLVSTFRYEG DDVNMMLARS EAKILHEKVS EKSYSDDDFI RILTTRSKAQ
Ann1Phloem      -------YEG DEVNFTLATQ EAK----------HYSDEDVI R---------
Ann2Phloem      ---------- ---------- ---------- ---------- ----------

AtAnn1          INATFNRYQD DHGEEILKSL EEGDDDDKFL ALLRSTIQCL TRPELYFVDV
AtAnn2          LGATLNHYNN EYGNAINKNL KEESDDNDYM KLLRAVITCL TYPEKHFEKV
Ann1Phloem      -------YQD EHGEEILKSL EEGDEDDKFL GLLR------ ----------
Ann2Phloem      ---------- ---------- ---------- ---------- ----------

AtAnn1          LRSAINKTGT DEGALTRIVT TRAEIDLKVI GEEYQRRNSI PLEKAITKDT
AtAnn2          LRLSINKMGT DEWGLTRVVT TRTEVDMERI KEEYQRRNSI PLDRAIAKDT
Ann1Phloem      ---------- ---------- ---------- ---------- ----------
Ann2Phloem      ---------- ---------- ---------- ---------- ----------

AtAnn1          RGDYEKMLVA LLGEDDA
AtAnn2          SGDYEDMLVA LLGHGDA
Ann1Phloem      ---------- -------
Ann2Phloem      ---VEDFLVS LLG----
```

Fig. 18 Amino acid sequence comparison between annexin1 (AtAnn1, At1g35720), annexin2 (AtAnn2, At5g65020) and partial amino acid sequences obtained from *B. napus* phloem sap analyses (see Fig.xxx) (Ann1Phloem, Ann2Phloem). The multiple alignment software ClustalW http://www.ch.embnet.org/software/ClustalW.html was used.

```
BnGRP10          ..MSEVEYRC FVGGLAWATG DAELERTFSQ FGEVIDSKII NDRETGRSRG
AtGRP8           ..MSEVEYRC FVGGLAWATN DEDLQRTFSQ FGDVIDSKII NDRESGRSRG
AtGRP7           MASGDVEYRC FVGGLAWATD DRALETAFAQ YGDVIDSKII NDRETGRSRG
BnPhloGRP        ---------- ---------- -------FSQ FGEVIDSK-- ----------

BnGRP10          FGFVTFKDEK SMKDAIDEMN GKELDGRTIT VNEAQSRG.G GGGGGRG..G
AtGRP8           FGFVTFKDEK AMRDAIEEMN GKELDGRVIT VNEAQSRGSG GGGGGRGGSG
AtGRP7           FGFVTFKDEK AMKDAIEGMN GQDLDGRSIT VNEAQSRGSG GGGGHRGGGG
BnPhloGRP        ---------- ---DAIEEFN GKELDGR--- ---------G GGGGGYG--G

BnGRP10          GGYGGRGGGG YGGGGGGYGD RRGGGG..YG SGGGGRGGGG YGSGGGGYG.
AtGRP8           GGYRSGGGGG YSGGGGGGY. ..SGG....G GGGYERRSGG YGSGGGGGGR
AtGRP7           GGYRSGGGGG YSGGGGSYG. ..GGGGRREG GGGYSGGGGG YSSRGGGGG.
BnPhloGRP        GG-------G YGGGGGGYGR ---------- ---------- ----------

BnGRP10          ..GGGGRRDG GGYGGGDGG. YGGGSGGGGW
AtGRP8           GYGGGGRREG GGYGGGDGGS YG..GGGGGW
AtGRP7           SYGGGRREGG GGYGGGEGGG YGGGSGGGGW
BnPhloGRP        ---------- ---------- ----------
```

Fig. 19 Amino acid sequence comparison between the GRP 10 protein from *B.napus* (BnGRP10, Q05966), GRP 8 from *A. thaliana* (AtGRP8, At4g39260, CAB80589), GRP 7 from *A. thaliana* (AtGRP7, At2g21660, AAL06943) and partial sequences obtained from *B. napus* phloem sap analyses (BnPhloGRP). The peptide sequence used for antibody production is marked in grey. Aligments were done using the multiple alignment software ClustalW http://www.ch.embnet.org/software/ClustalW.html.

```
BnMA_Myr2A_      MKLLHGLALV FLLAAASCKA DEEITCEENN PFTCSNTDIL SSKNFGKDFL
BnMB_Myr1A_      MKHLG.LILA FLLALATCKA DEEITCEENL PFKCSQPDRL NSSSFEKDFI
BnMB             MKLLHGLALV FLLAAASCKA DEEITCEENN PFTCSNTDIL SSKNFGKDFI
BnMC             MKFRGLDLIV FLLAVVSCKA NKEITCEENE PFTCNNTDRL NSKGFPKDFI
AtMyr            MKLLM.LAFV FLLALATCKG D.EFVCEENE PFTCNQTKLF NSGNFEKGFI
BnPhloemMyr      ---------- ---------- -------ENE PFTCQNTDQL SSK----DFN

BnMA_Myr2A_      FGVASSAYQA ....CRGVNV WDGFSHRYPE KSGSDLKNGD TTCESYTRWQ
BnMB_Myr1A_      FGVASSAYQA .CCLGRGLNV WDGFTHRYPN KSGPDHGNGD TTCDSFSYWQ
BnMB             FGVASSAYQI EGGRGRGVNV WDGFSHRYPE KAGSDLKNGD TTCESYTRWQ
BnMC             FGVSSAAYQI EGGRGRGLNI WDGFTHRFPE KGGSDLGNGD TTCESYTMWQ
AtMyr            FGVASSAYQV EGGRGRGLNV WDSFTHRFPE KGGADLGNGD TTCDSYTLWQ
BnPhloemMyr      FGVASAAYQV EGGR------ ---------- -GGSDLGNGD TT--------


BnMA_Myr2A_      KDVDVMGELN ATGYRFSFAW SRIIPKGKVS RGVNQGGLDY YHKLIDALLE
BnMB_Myr1A_      KDIDVLDELN ATGYRFSIAW SRIIPRGKRS RGVNKDGINY YHGLIDGLID
BnMB             KDVDVMGELN ATGYRFSFAW SRIIPKGKVS RGVNQGGLDY YHKLIDALLE
BnMC             KDIDIMDEMN ATGYRFSFAW SRIIPKGKVS RGVNKGGLEY YHRLIDGLIA
AtMyr            KDIDVMDELN STGYRFSIAW SRLLPKGKRS RGVNPGAIKY YNGLIDGLVA
BnPhloemMyr      ---------- ---------- ---------- ---------- ----------

BnMA_Myr2A_      KNITPFVTLF HWDLPQTLQD EYEGFLDRQI IQDFKDYADL CFKEFGGKVK
BnMB_Myr1A_      KGITPFVTLF HWDLPQVLQD EYEGFLDPQI IHDFKHYANL CFQEFGHKVK
BnMB             KNITPFVTLF HWDLPQTLQD EYEGFLDRQI IQDFKDYADL CFKEFGGKVK
BnMC             KNITPFVTLY HWDLPQTLQD EYEGFLNRQV IEDFRDLADL CFKEFGGKVK
AtMyr            KNMTPFVTLF HWDLPQTLQD EYNGFLNKTI VDDFKDYADL CFELFGDRVK
BnPhloemMyr      ---------- ---------- ---------- ---------- ----------

BnMA_Myr2A_      HWITINQLYT VPTRGYAVGT DAPGRCSPMV DTKHRCYGGN SSPEPYIVAH
BnMB_Myr1A_      NWLTINQLYT VPTRGYGAGS DAPGRCSPMV DPT..CYAGN SSTEPYIVAH
BnMB             HWITINQLYT VPTRGYAIGT DAPGRCSPMV DTKHRCYGGN SSTEPYIVAH
BnMC             NWLTINQLYS VPTRGYSTGA DAPVRCSPKV DAR..CYGGN SSTEPYIVAH
AtMyr            NWITINQLYT VPTRGYALGT DAPGRCSPKI DVR..CPGGN SSTEPYIVAH
BnPhloemMyr      ---------- ---------- ---------- ---------- ----------
```

```
BnMA_Myr2A_      NQLLAHATVV DLYRTKYKF. QKGKIGPVMI TRWFLPFDES DPASIEAAER
BnMB_Myr1A_      NQLLAHATVV DLYRKNY... ..S.IGPVMI TRWFLPYNDT DPDSIAATER
BnMB             NQLLAHATVV DLYRTKYKF. QKGKIGPVMI TRWFLPFDES DPASIEAAER
BnMC             NQLLAHTAVV NLYRTKYRF. QRGRIGPVMI TRWFLPFDET NKASIDAAER
AtMyr            NQLLAHAAAV DVYRTKYKDD QKGMIGPVMI TRWFLPFDHS Q.ESKDATER
BnPhloemMyr      ---------- ---------- ---------- ---------- ----------

BnMA_Myr2A_      MNQFFHGWYM EPLTKGRYPD IMRQIVGSRL PNFTEEEAEL VAGSYDFLGL
BnMB_Myr1A_      MKEFFLGWFM GPLTNGTYPQ IMIDTVGERL PSFSPEESNL VKGSYDYLGL
BnMB             MNQFFHGWYM EPLTKGRYPD IMRQIVGSRL PNFTEEEAEL VAGSYDFLGL
BnMC             MKEFFLGWYM EPLTRGRYPD IMRRMVGNRL PNFTEAEARL VAGSYDFLGL
AtMyr            AKIFFHGWFM GPLTEGKYPD IMREYVGDRL PEFSETEAAL VKGSYDFLGL
BnPhloemMyr      ---------- ---------- ---------- ---------- ----------

BnMA_Myr2A_      NYYVTQYAQP KPNPYP..SE THTAMMDAGV KLTYDNSRGE FLGPLFVEDE
BnMB_Myr1A_      NYYVTQYAQP SPNPVH..WA NHTAMMDAGA KLTFRGNSDE ..TK......
BnMB             NYYVTQYAQP KPNPYP..SE THTAMMDAGV KLTYDNSRGE FLGPLFVEDK
BnMC             NYYATQFVQP TPNPLPVTSE RYTAMMDPGT RLTFVNSRGE KTGPLFEELK
AtMyr            NYYVTQYAQN NQTIVP..SD VHTALMDSRT TLTSKNATGH APGPPFN...
BnPhloemMyr      ---------- ---------- ---------- ---------- ----------

BnMA_Myr2A_      VNGNSYYYPK GIYYVMDYFK TKYGDPLIYV TENGFSTPSS ENREQAIADY
BnMB_Myr1A_      ...NSYYYPK GIYYVMDYFK TKYYNPLIYV TENGISTPGN ETRDESMLHY
BnMB             VNGNSYYYPK GIYYVMDYFK TKYGDPLIYV TENGFSTPSS ENREQAIADY
BnMC             G.GNSYYYPP GIYYVMDYFT TKYRNPLIYI TESGFSTSGD QTRQEAVADS
AtMyr            ..AASYYYPK GIYYVMDYFK TTYGDPLIYV TENGFSTPGD EDFEKATADY
BnPhloemMyr      ---------- ---------- --YSNPLIYI TENG------ ----------

BnMA_Myr2A_      KRIDYLCSHL CFLRKVIKEK GVNVRGYFAW ALGDNYEFCK GFTVRFGLSY
BnMB_Myr1A_      KRIEYLCSHL CFLSKVIKEK HVNVKGYFAW SLGDNYEFDK GFTVRFGLSY
BnMB             KRIDYLCSHL CFLRKVIKEK GVNVRGYFAW ALGDNYEFCK GFTVRFGLSY
BnMC             KRIDYLCSHL CFLRKVIMEK RVNIKGYFAW ALGDNYEFGK GFTVRFGLSY
AtMyr            KRIDYLCSHL CFLSKVIKEK NVNVKGYFAW SLGDNYEFCN GFTVRFGLSY
BnPhloemMyr      ---------- ---------- ---------- ---------- ----------

BnMA_Myr2A_      VNWEDLD.DR NLKESGKWYQ RFINGTVKNS AKQDFLRSSL SSQSQ.KKKL
BnMB_Myr1A_      IDWNNVT.DR DLKLSGKWYQ KFISPAIKNP LKKDFLRSSL TFE.K.NKKF
BnMB             VNWEDLD.DR NLKESGKWYQ RFINGTVKNA VKQDFLRSSL SSQSQ.KKRF
BnMC             VNWTDVS.DR NLKDSGKWYQ RFINVTTKIT AHQDFLRSGL SFEDK.MKTL
AtMyr            VDFANITGDR DLKASGKWFQ KFINVTDEDS TNQDLLRSSV SSKNRDRKSL
BnPhloemMyr      ---------- ---------- ---------- ---------- ----------

BnMA_Myr2A_      ADA
BnMB_Myr1A_      EDA
BnMB             ADA
BnMC             TDA
AtMyr            ADA
BnPhloemMyr      ---
```

Fig. 20 Amino acid sequence comparison between different myrosinase isoforms from *B. napus* (BnMA_Myr2A - Z21978; BnMB_Myr1A - Z21977; BnMB - X60214; BnMC - X79080), the most homologous myrosinase from *A. thaliana* (AtMyr - AY090382, At5g26000) and the partial sequences obtained from *B. napus* phloem sap analyses (BnPhloemMyr). The multiple alignment software ClustalW http://www.ch.embnet.org/software/ClustalW.html was used.

The application of the presented criteria led to the selection of knockout lines for annexin 1, annexin 2, myrosinase and the glycine-rich RNA binding protein 7, details about the lines are presented in Table 2. Annexin 1 was identified by as many as 10 partial sequences from 2 DE/MS analysis and 4 partial sequences were obtained for

annexin 2. It was possible to distinguish annexin 1 and 2 from these partial amino acid sequences (Fig. 20). Knockout lines for both of these proteins were available. 5 different partial sequences obtained from 4 spots on 2 DE identified as myrosinase showed high similarity to one of the *A. thaliana* myrosinases (At5g26000) and the knock out line for this *Arabidopsis* gene was selected. It was however not possible to assign the detected protein to any specific *B. napus* myrosinase type (Fig. 22). Sequences obtained for the glycine-rich RNA binding protein, showed highest homology to a GRP10 from *B. napus* but also showed significant similarity to *A. thaliana* GRP 8 and GRP 7 (Fig. 21). Since no knock-out line with the T-DNA insertion within the coding sequence of the more homologous GRP 8 was available, a line with an insert in the GRP 7 gene, which showed also high sequence similarity, was selected.

3.5.2.2 Screening for homozygous lines

From the employed knock-out mutants the homozygous lines were selected by PCR. PCR analyses were performed using primer pairs specific for the tested genes and one primer specific for the T-DNA sequence. Gene specific primer pairs were designed and 35 plants were screened for each mutant (nucleotide sequences of the primers are available in the Table II in appendix). Two separate PCR reactions were performed to test each line. A line was regarded as homozygous when no product occurred in the PCR reaction with gene specific primers while a product from the reaction with one gene specific primer and a T-DNA sequence specific primer was present (Fig. 23). Several homozygous lines were selected for each gene and were used for further investigations (see Table 2).

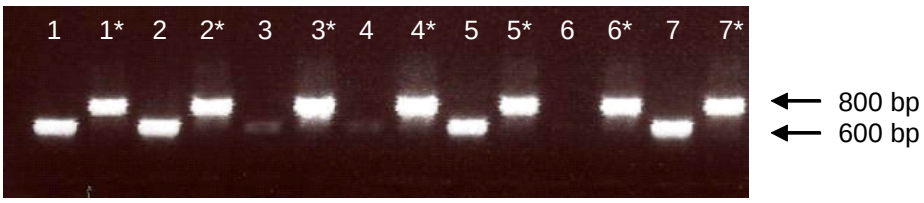


Fig. 21 Screening of an example *Arabidopsis* mutant for homozygous lines. PCR amplified fragments from seven T2 generation plants were resolved on an agarose gel. Each mutant line (presented in two neighbouring slots) was tested with two primer pairs. One primer pair for the detection of T-DNA insertion resulting in the production of a larger fragment (slots 1-7) and a second for the detection of the uninterrupted gene, resulting in a shorter fragment (slot 1*-7*). When both products appeared the respective plant was classified as heterozygous (line 2, 5 and 7), when only one product was detected the plant was either assigned as homozygous (3, 4 and 6) or as a wild type (line 1).

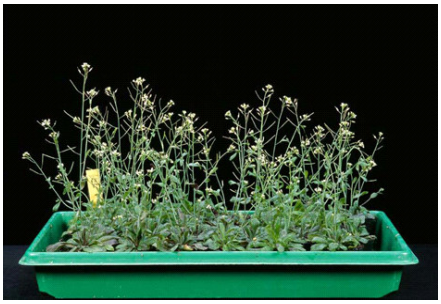
Protein	Accession No	No of sequences (ESI-MS)	T-DNA insert	Mutant name
Annexin1	At1g35720	10	exon	SALK_
Annexin2	At5g65020	4	exon	SALK_
Myrosinase	At5g26000	5	intron	GABI-Kat 693D05
Glycin-rich RNA binding protein 7	At2g21660	3	intron	SALK_113110

Table 2 List of the proteins investigated by *A. thaliana* knockout mutant analyses. The obtained partial amino acid sequences (number of different sequences obtained from several spots identifying one protein is presented) used for identifications are accessible as supplemental data. Mutant names and deduced T-DNA insertion sites are listed in the right columns.

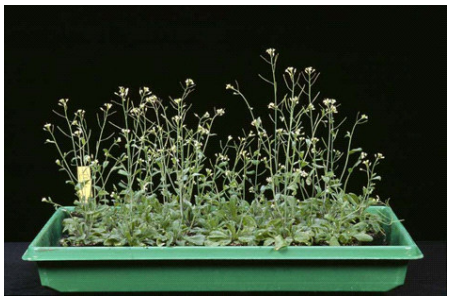
Annexin 1 and annexin 2 mutants were a gift from Prof. Okhmae Park from Kumho Life and Environmental Science Laboratory, Gwangju, Korea and the obtained seeds were already homozygous (Lee et al., 2004).

The selected homozygous lines were subsequently planted to investigate the phenotypes. Plants were grown under the same, controlled conditions and were randomly mixed in the trays. 7 weeks after germination plants were ordered in the

trays according to the mutant line and visually compared to the WT (Fig. 24). No visible changes in phenotype were however observed for any of the mutants.

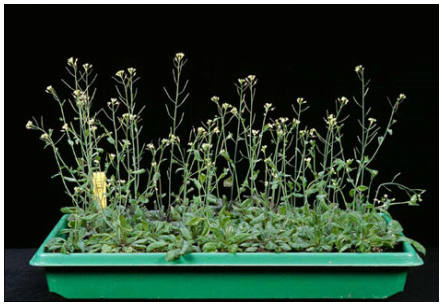


WT

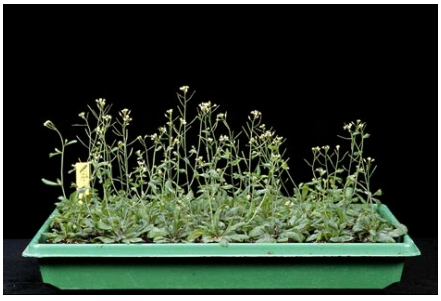


GRP 7

Annexin 1



Annexin 2



Myrosinase

Fig. 22 Comparisons of 5 week old *Arabidopsis* knock out mutants and a wild type plants (WT). Plants were grown randomly mixed in the trays under the same, controlled conditions. No obvious differences in the phenotypes could observed in any of the KO lines when compared to the WT (GRP 7: glycine-rich RNA-binding protein 7).

3.5.3 Grafting experiments

The first attempts towards a functional characterisation of the investigated proteins using T-DNA insertion lines were performed on the GRP 7 mutants. Wild type plants and the GRP 7 KO lines were analysed by western blots to verify the absence of protein expression in the homozygous plants. The protein transportability was examined by grafting experiments combined with western blot studies. For the grafts wild type *A. thaliana* plants were used as a stock and KO lines served as a scion. Antibodies against GRP 7 described in the previous section (Fig. 16) were used to test if GRP 7 appeared in the KO scions of the grafts, which would indicate phloem transport of this protein. To achieve this, young inflorescence stems of 6 week-old *Arabidopsis* plants were cut and grafted according to the method described by Rhee & Somerville (2004) using silicon collars as a support of the junction sites (see Fig. 25). The grafted plants were kept for 2-4 days in high humidity conditions and afterwards the humidity was gradually decreased. Functional junctions could be determined by visible growth of the scion (Fig. 25). Approximately 20% of the grafts were successful in each experiment, independently of the grafted mutant. Stem extracts were prepared from both stock and scion parts of functional grafts.

Fig. 23 10 days old *A. thaliana* graft. Annexin1 mutant (scion) was grafted onto a WT Col0 stock using collar a (indicated by an arrow) to support the graft junction.

The anti GRP antibody produced on the peptide common for *B. napus* GRP 10 and *A. thaliana* GRP 7 were shown to react with phloem proteins from rape but also with stem extract from *Arabidopsis* (Fig. 16). The protein marked with the antibody was in the similar size for both plants and the signal was absent from *Arabidopsis* leaf tissue (data not shown). However, more than one protein band was marked by this antiserum when a higher protein amount was loaded on the gel (Fig. 26), indicating that more than one GRP protein is present in the phloem sap of rape and in the stem of *Arabidopsis*.

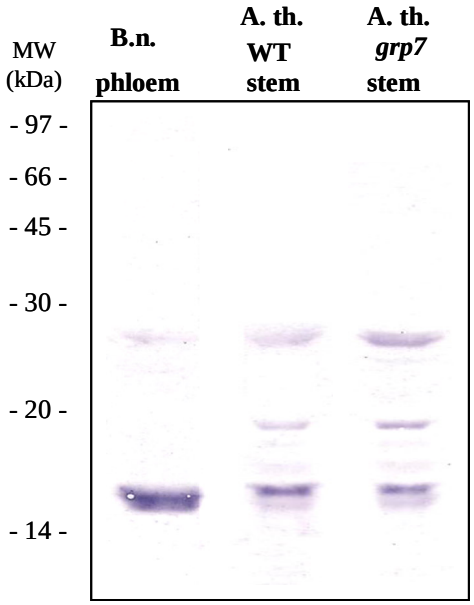


Fig. 24 Detection of glycine-rich RNA-binding proteins (GRP) in stem extracts of *A. thaliana* (A. th.) and phloem sap of *B. napus* (B. n.). Protein extracts from flower stems of wild type (WT) and glycine-rich RNA-binding protein 7 mutant (*grp7*) were transferred to nitrocellulose and incubated with anti-GRP peptide antibody. No signal was observed with the leaf extracts from *A. thaliana*

Additionally, GRP7 KO lines and wild type *Arabidopsis* showed the same pattern when blotted with anti GRP antibodies. Moreover, it seemed that the produced antiserum cross reacted with other GRP proteins from both Arabidopsis stem extract and *B. napus* phloem sap.

4 Discussion

The work of this thesis was focused on the identification and partial characterisation of novel phloem proteins in different plant species.

For protein identification two independent approaches were undertaken. Firstly, expression library screening using phloem protein-specific antisera and secondly, protein separation on 2 DE followed by ESI-Q-TOF peptide sequencing and subsequent database similarity searches were performed. Compilation of the applied techniques allowed the identification of a number of new phloem sap proteins from every tested species. Once the proteins were identified, it was intended to functionally characterise some of them by a reverse genetic approach using T-DNA insertion lines.

4.1 Collection of phloem samples from cucumber, pumpkin and rape plants

Collection of pure phloem sap samples is a crucial prerequisite to perform protein analyses. The phloem sampling procedure (modified exudation) used in this study could cause the contamination of samples with destroyed cells from surrounding tissues by the incisions necessary to induce phloem exudation. To verify the purity of collected phloem samples exudates were tested for the presence of the rubisco protein. Rubisco, a photosynthetic protein highly expressed in chloroplasts (Miziorko and Lorimer, 1983), was not expected to be present in phloem sap, because the residual SE plastids are not photosynthetically active (Behnke, 1991). It was demonstrated that the small, nuclear encoded, subunit of rubisco could not be detected on western blots of *B. napus* phloem samples (Fig. 2). Additionally the measured soluble sugar concentrations in the collected samples where sucrose was dominating and hexoses were at very low levels, give a confirmation of the exudates' origin (Fig. 3). In *B. napus,* as in other apoplasmic phloem loaders, sucrose is the major transported sugar in the phloem in contrast to the total source leaf tissue of *B. napus* where glucose is dominating sugar (King et al., 1997).

Results from all the performed experiments consistently indicate a reasonably low contamination of samples with broken cells. Assuming that all glucose detected is

caused by contamination, the estimated percentage contamination would be lower than 1.5 %. The used phloem exudation technique was tested previously in *B. napus* and as reported in Kapitza *et al.* (2004) and Giavalisco *et al.* (2005) the protein composition detectable on 1 DE and 2 DE remained largely unchanged during the period of exudation (several hours) and was reproducible between different sets of plants. Also in cucurbits it has been shown earlier that the modified exudation technique resulted in samples free from other tissue contamination and of stable protein composition throughout the sampling period (Walz, 2002).

Taken together, these results indicate that the modified exudation technique used here allows the collection of reasonably pure phloem samples from the three plant species under investigation.

However, it cannot be excluded that some of the proteins originate from companion cells surged in by the turgor loss induced during sampling. The possible contaminations can be also derived from SE plastids that can store proteins and are destroyed by mechanical damage, as hypothesized earlier (Eschrich and Heyser, 1975; Knoblauch and van Bel, 1998). To fully document the lack of contaminations the exact *in vivo* localization would have to be evaluated for every individual protein. It was demonstrated before, however, that samples obtained with the minimal invasive aphid stylet technique show similar protein patterns and concentrations like phloem exudates (Fisher et al., 1992; Barnes et al., 2004). Further, the obtained protein patterns were demonstrated to be constant over hours or even days of exudation (Fisher et al., 1992; Sakuth et al., 1993). Additionally, both the results from sugar measurements and western blot analysis indicate that the collected phloem samples contain relatively pure phloem sap and that contamination from broken surrounding cells appears to be very low.

4.1.1 Metabolites in the phloem sap of *B. napus*

To further characterise phloem samples collected from rape metabolite profile of the SE exudates was measured by GC MS. As previously described, phloem sap contains wide range of metabolites (Richardson and Baker, 1982; Lohaus et al., 2000; Lohaus and Moellers, 2000; Arlt et al., 2001; Fiehn, 2003) that can be transported over long distances or can be responsible for maintaining the functionality of the phloem. The available data on *B. napus* phloem metabolites

provides information mainly on the amino acid composition within leaf SE sap (Lohaus and Moellers, 2000).

Using the GC-MS approach in this work, as a method that facilitates simultaneous detection of very many compounds with additional high sensitivity, it was possible to identify more than 70 different metabolites in stem phloem sap and 58 in total inflorescence stem extracts of *B. napus*.

For *B. napus* like for other apoplasmic phloem loading plant species, sucrose is the dominating transport form of sugars. Consistent with this, sucrose was the most abundant sugar measured in phloem sap of *B. napus*. The sucrose concentration in the phloem was with approximately 0.6 Mol (Fig. 3) similar to the previously reported sucrose levels in sap from *B. napus* leaves, being in the range of 0.9-1.3 Mol depending on the cultivar (Lohaus and Moellers, 2000). Additionally, very low levels of glucose and fructose are consistent with the results from phloem exudates of other species (Geigenberger et al., 1993; Lohaus et al., 1998). No information on soluble sugar concentrations in the inflorescence stems of *Brassicaceae* or any other higher plant species was found to be published so far. In the measured samples of flower stem, glucose was the most concentrated sugar followed by fructose and the approx. 20 times less abundant sucrose. A similar distribution of soluble sugars was found in the midrib of source leaves in tobacco plants (Geigenberger et al., 1996). Further, high glucose, low fructose and significantly lower sucrose concentrations were measured in source leaves of *B. napus* (King et al., 1997). For most plants, however, sucrose is the most abundant sugar in mature leaves (Klages et al., 1998; Buchanan et al., 2000).

The next most abundant substances within the group of sugars and sugar alcohols in phloem sap was glucose-6-phosphate followed by myo-inositol and fructose-6-phosphate (Fig. 4 B). Sugar alcohols are known to be transported in the phloem of several species as an additional source of non-reducing carbohydrates but also supplying sinks that have little or no capacity to synthesise sugar alcohols (Loesher and Everard, 1996). Their concentration and distribution, however, differ considerably between species (Ziegler, 1975). Myo-inositol was also the most abundant sugar alcohol detected in stem extracts and its level was drastically higher than of the other sugars and sugar alcohols in the stem. Interestingly, myo-inositol was also higher in the stem than in phloem sap (Fig. 7 B). In contrast, hexose phosphate levels were much higher in the phloem than in the stem (Fig. 7 B). Many different hexose

phosphates were identified already in various plant species in the phloem at relatively high levels (see for review (Ziegler, 1975)). It is very likely that these organic phosphorous-containing compounds are involved in metabolic processes inside the phloem rather than being just transported to sink organs as substrates for their metabolism. This idea is supported by a number of enzyme activities (Lehmann, 1973a, 1973b) and potential metabolic proteins found in vascular exudates (Hayashi, 2000; Haebel and Kehr, 2001; Walz et al., 2002; Barnes et al., 2004; Walz et al., 2004; Giavalisco et al., 2005). Interestingly, several sugar and sugar derivatives were detected exclusively in the phloem samples (rhamnose, glycerol-1-phosphate, myo-inositolo-1-phosphate), while others were found only in the stem (gentobiose, raffinose, maltitol). This observation could confirm the selectivity of sugar transport in the phloem. The levels of these sugars were, however, relatively low in both tissues.

As in many plants, glutamine was the major amino acid also in *B. napus* phloem samples (Fig. 4 A). Unexpectedly, the most abundant amino acid detected in the stem and one of the major amino acids in the phloem was oxoproline (pyroglutamic acid) (Fig. 4 A, 5 A). These high amounts of this proline derivative are most likely caused by an arteficial conversion of the temperature sensitive amino acids glutamate and glutamine to pyroglutamic acid during sample preparation (Persson and Nasholm, 2001). This would also explain the lower than expected levels of glutamine and glutamate in stem and phloem sap samples. The high amount of serine that was detected is in accordance with previous data on *B. napus* (Lohaus and Moellers, 2000; Seiffert et al., 2004). The high levels of proline measured in both phloem and stem samples were not reported so far in oil seed rape but were found in the phloem of other plant species (Hocking, 1980; Richardson et al., 1982). High levels of proline, valine and isoleucine in the phloem and stem samples might be an indication that the investigated plants were stressed (Girousse et al., 1996). This seems however unlikely, since the sampled rape plants were grown in the greenhouse under controlled light and temperature conditions and with regular automatic water supply. From the proteogenic amino acids only histidine was not detected in both tested tissues. Histidine has been previously shown to be under the detection limit also in the phloem sap of *Brassicaceae* leaves (Lohaus and Moellers, 2000). Generally, the relative levels of total amino acids as well as their distribution were similar for phloem and stem samples. Interestingly, high levels of putrescine were measured in the phloem sap while in the stem it was hardly detectable (Fig. 7

A). It can be speculated that this polyamine precursor is involved in phloem metabolism rather than being simply transported into sink organs. Putrescine can be formed in the process of amino acid breakdown and could serve in the phloem as a polyamine precursor. The very low levels of cystein and homocysteine measured in this study and the previous detection of several enzymes responsible for conversion of cysteine and homocysteine into S-adenosylmethinone (Giavalisco et al., 2005) in the phloem seems to be supportive for the idea of amino acid metabolism in the SE sap. S-adenosylmethionine itself has been shown to be one of the major components of phloem sap in many plants (Bourgis et al., 1999). However, this compound was not included in the list of analysed metabolites in the presented measurements.

It has been previously shown that phloem sap contains a number of organic and inorganic acids that can be either transported or be involved in different metabolic pathways active in the phloem (Kennecke et al., 1971; Geigenberger et al., 1993; Hancock et al., 2003). In this work 22 substances from this group of metabolites were identified in both phloem and stem tissue. Their distribution in both tissues looked similar (Fig. 7 C). The highly abundant pool of D- and L-ascorbic acid and dehydroascorbate correlates with previous findings from phloem sap of different species where not only the presence of ascorbic acid (AsA) but also the activity of many enzymes involved in AsA biosynthesis and conversion was demonstrated (Hancock et al., 2003; Giavalisco et al., 2005). Their role is most likely to prevent SE from oxidative damage caused by reactive oxygen species that are produced at higher levels upon environmental and biotic stresses (Scandalios, 1997; Walz et al., 2002). Malate, citrate and succinate have also been already described as SE exudate compounds in many plants (Ziegler, 1975; Fiehn, 2003).

As a summary, the GC-MS metabolic profile of *B. napus* phloem sap presented in this work reflects the metabolite composition of a typical apoplasmic phloem loader. This confirmed previous studies on phloem sap metabolite composition (Weibull et al., 1990; Winter et al., 1992; Lohaus et al., 1995; Lohaus and Moellers, 2000; Fiehn, 2003) and reflects that the GC-MS method is suitable for SE exudates analyses. Morover differential metabolite distribution between phloem sap and stem extract together with measured phloem metabolite profile typical for apoplasmic phloem loader indicate lack of significant sample contamination from the surrounding cells as discussed before.

4.2 Phloem protein identification

Although in mature sieve elements transcription and translation can probably not take place because nuclei and ribosomes degenerate during differentiation, it was demonstrated before that several hundred polypeptides can be separated from phloem exudates of different species by gel eletrophoresis (Fisher et al., 1992; Walz et al., 2004; Giavalisco et al., 2005). From these phloem proteins only a limited number was identified until now (Crawford and Zambryski, 1999; Hayashi, 2000; Haebel and Kehr, 2001; Walz et al., 2004) and the functions of most of them still remain unknown.

4.2.1 Screening of cucumber, pumpkin and rape expression libraries

Most recent data about phloem proteins come from *Cucurbitaceae* and *R. communis* and have been derived mainly from identification of partial peptide sequences (Kehr et al., 1999; Haebel and Kehr, 2001; Walz et al., 2002; Barnes et al., 2004; Walz et al., 2004) and therefore do not contain information about the complete sequences of the proteins. To study their functionality using forward and/or reverse genetic methods, full length sequences of phloem proteins have to be identified. Within this thesis three separate expression libraries were constructed and screened with phloem protein specific antisera to obtain full length cDNA sequences of phloem proteins from *C. maxima*, *C. sativus* and *B. napus*.

The phloem protein-specific antisera used for screening the expression libraries showed very high specificity for the phloem proteins they were raised against (Fig. 8). No cross reactivity could be observed, neither between phloem proteins from different species, nor between leaf and phloem polypeptides within one plant species (Fig. 8). These surprising observations raised the need for further studies on the employed antibodies. Thus, immunoglobulins that bound to three distinct phloem protein bands on 1 DE were affinity purified from the complete antiserum and were separately tested against complete phloem sap proteins. It was demonstrated that each portion of purified antibodies react with several protein bands on the western blot. The experiments showed that the tested antibodies seemed to be produced mainly against a similar or the same epitope that is shared by different phloem proteins within one species (Fig. 11). Such epitope might be formed, for example, of a highly antigenic amino acid sequence (Atassi, 1975), post-translational amino acid

modifications (mainly glycosylations (Ogawa et al., 1996)), or of specific molecules bound to the amino acids (nucleic acids, haptens (Charles and Clayman, 1989)). It was excluded that antibodies were produced on glycosylation or phosphorylation sites on phloem proteins (Fig. 10) by in gel staining of glycosylated and phosphorylated proteins followed by comparison to the protein patterns on western blots. As previously demonstrated, many proteins contained in phloem exudates can be glycosylated (Walz, 2002) and phosphorylated (Nakamura et al., 1993; Avdiushko et al., 1997) what was confirmed for phloem proteins from *Cucurbitaceae* and *B. napus* in this work. Also within the sequences identified from screening the libraries no common amino acid sequence that could be considered as an antigenic epitope was found. However, it can not be excluded that such a sequence exists in some of the phloem proteins. To identify proteins that react with the antiserum, immunoprecipitation of the antigenic phloem polypeptides was performed (Fig. 12). Proteins precipitated using antiserum were separated on 1 DE and could be visualised by sensitive silver staining as well as on western blots. Unfortunately, these polypeptides could not be visualised with mass spectrometry-compatible coomassie blue staining what disabled MS analyses and therefore no sequence information of immunoprecipitated proteins could be obtained. The low protein amounts after immunoprecipitation hampered also an analysis with MALDI-TOF which was planned to investigate other specific molecules (possible haptens) that could bind to selected phloem proteins forming an antigenic epitope. Hence, the question about the nature of the antigenic epitope(s) remained not answered. However it was expected that the employed polyclonal antibody mixture would additionally recognise other phloem proteins that do not contain this one common epitope. For that reason the antisera were anyhow used to screen the expression libraries. To exclude the possibility that false positives were isolated from the libraries, phloem localisation of several of the identified proteins was additionally confirmed by immunolocalisation experiments (Fig. 16).

The constructed expression libraries contained a large amount of clones (described as plaque forming units) and the cDNA fragments were in the size of approximately 1 kb (Fig. 13), which allows expecting full length sequences among them. However, considering the fact that mesophyll cells make up about 75% of the total leaf cells (Winter et al., 1994; Leidreiter et al., 1995) and phloem cells constitutes only a part of the remaining 25% it was born in mind that the libraries might contain only a relatively

small representation of phloem specific transcripts. Hence, cucumber and pumpkin libraries were first tested for the presence of the cDNA of a known cucumber phloem sap protein, the fruit ripening and wound induced CSF-2 cDNA (Haebel and Kehr, 2001; Walz et al., 2004), using a gene-specific probe. From both libraries full length clones almost identical (pumpkin) and identical (cucumber) to the CSF-2 sequence present in the database (Suyama et al., 1999) were identified (Fig. 14). This experiment indicated that the created expression libraries do contain phloem specific full length clones that can be isolated, sequenced and compared with the database entries.

After screening the libraries with specific antisera, 14 different proteins were identified. The relatively low number of identified proteins in comparison to the number of selected positive clones (120 from all three libraries) was caused firstly by a high number of selected inserts that were too short to assign them as putative full length clones and were therefore discriminated; secondly, a large number of very similar sequences that showed no homology to any database entry were obtained after sequencing. Thus, it seemed likely that many antibodies within the antisera used for screening were mainly mono specific (accordingly to the results obtained with immunoprecipitation).

However, the employed antisera allowed to clone and identify some proteins known from cucumber phloem in cucumber phloem samples (the 17 kDa phloem lectin) and also an abundant phloem sap protein in rape phloem samples (thioredoxin h) (Table 1). These findings confirm earlier reports on the presence of these proteins in the phloem exudates. As described by (Dinant et al., 2003; Walz et al., 2004) a large number of lectins is present in the phloem of cucurbits with a 17 kDa lectin known to be one of the major phloem sap proteins in cucumber. Additionally, one of the well characterised and most abundant phloem lectins from *Cucubitaceae,* namely PP2, had also been found in rape phloem samples (Fig. 15) by Giavalisco *et al.* (2005). PP2 together with PP1 is thought to function in stress and pathogen response what is likely to be connected with their abilities in sealing of sieve pores upon wounding (Alosi, 1988). PP2 was also described to be able to translocate within sieve elements (Golecki et al., 1999) and, because of its RNA binding facilities, suggested to play a role in the long-distance transport of viroids in cucumber (Gomez and Pallas, 2001; Owens et al., 2001). Despite the fact that 17 and 26 kDa lectins share only 40% to 50% of amino acid identity with the PP2 protein from *C. maxima* (Dinant et al., 2003)

their functions might be similar. The 17 kDa lectin from melon was described to be able to bind RNA and translocate it over long distances (Gomez et al., 2005) and aspects of RNA transport in the phloem are discussed in more details in the following section. Interestingly, it was also shown that the expression of the *C. sativus* 17 kDa lectin was down regulated by cytokinins (Toyama et al., 1995) that are transported in the phloem (Hoad, 1995) and together with auxin are involved in phloem differentiation (Aloni, 1995).

Thioredoxin h identified in the phloem sap of *B. napus* has previously been described as a major protein in rice phloem sap (Ishiwatari et al., 1995). This protein can regenerate proteins damaged by oxidative stress and can be part of a redox-regulating protein system that was demonstrated to be present in the phloem (Walz et al., 2002; Walz et al., 2004). Reactive oxygen species are produced by the plant as a general reaction to stress and pathogen attack. To protect endogenous components from oxidative damage, the presence of scavenging systems composed of proteins and metabolites is essential. Within this work thioredoxin h has also been identified in phloem samples from *B. napus* using the alternative approach, 2 DE protein separation followed by ESI-Q-TOF sequencing, where two abundant spots could be assigned to thioredoxin h (Fig. 15).

Considering the fact that most proteins within SEs are most likely produced in CC and transported to SE (Oparka, 2004) the presence of a system to maintain the stability and translocation of phloem sap proteins seems to be indispensable. It was proposed that proteins with chaperone activity are involved in trafficking through plasmodesmata and refolding of phloem proteins on the SE site (Balachandran et al., 1997). In this studies a protein with high homology to low molecular weight heat shock protein 1 (LHS1) was identified (Table 1). Several heat shock proteins have already been found in SE exudates of different species (Schobert et al., 1998; Barnes et al., 2004; Giavalisco et al., 2005). Additionaly, rubisco activase, a protein identified from the *C. sativus* library can be classified as a member of the AAA^+ protein group (ATPases associated with diverse cellular activities) that constitutes a large variety of proteins with chaperone-like functions (Ogura and Wilkinson, 2001; Houtz and Portis, 2003). AAA^+ proteins can be involved in protein assembly and regulatory breakdown of protein complexes. Rubisco activase appears to promote and maintain the catalytic activity of rubisco (Portis, 2003) this involves also a role in the response of photosynthesis to temperature stress (Feller et al., 1998). Other

proteins of AAA^+ group have also been found in the phloem by (Giavalisco et al., 2005) and could function as an alternative route to translocate proteins from CC into SE. The presence of rubisco activase in the phloem sap was further confirmed by its identification on 2 DE (Fig. 15) and by its detection on western blots (Fig. 16). The employed anti rubisco activase antibodies detected two bands of this protein in phloem samples from rape. The size of the detected rubisco activase in the phloem as well as multiple signals observed in the leaf extracts agrees with previous data (Feller et al., 1998).

Many of the currently identified phloem proteins can be related to stress and defence reactions (Hayashi, 2000; Walz et al., 2004). Dehydrin, a protein involved in the stress response in plants (Borovskii et al., 2002) was identified in this study from the cucumber and pumpkin libraries (Table 1). Its presence in the phloem has also been verified by western blot analyses (Fig. 16, 17) where dehydrin was detected in the phloem sap of all three tested species. In the phloem samples from rape, anti-dehydrin antibodies identified a large number of proteins of different sizes. As previously described by (Close et al., 1993), the size of dehydrins ranges from 15 to about 150 kDa within different plant species. Dehydrins are a large group of proteins called also LEA (late embryogenesis-abundant) or RAB (responsive to abscisic acid - ABA). Studies on *Arabidopsis* and tobacco dehydrins show their accumulation under ABA, cold and drought stress in almost every tissue while in unstressed plants differential expression of dehydrins was observed (Rizhsky et al., 2002). This indicates that dehydrins may be specialised in their functions, however their role is accentuated under stress conditions (Nylander et al., 2001). Therefore, it can be speculated that dehydrins can be a part of an existing defence system in the phloem.

Additionally, from the expression libraries of cucumber and rape 14-3-3-like proteins were identified (Table 1). Western blots with 14-3-3 specific antiserum confirmed the presence of this protein in the phloem of rape and pumpkin but not in the phloem of cucumber (Fig. 16). Western blots showed that the phloem sap of rape similarly to leaf extracts contains two probably monomeric isoforms of 14-3-3 in the size of previously described plant 14-3-3 proteins (Wilczynski et al., 1998), but also a dimeric form in the size of approx. 60 kDa. In the phloem sap of *C. maxima* only a potential dimer of 14-3-3 was detected. It is known from crystallographic studies that 14-3-3 proteins appear in a dimeric form (Aitken, 1996) and functional dimers of 14-3-3 are also suggested to be present in plants (May and Soll, 2000). Interestingly, the

employed antibodies did not recognise a 14-3-3 protein in phloem samples of *C. sativus* from which the 14-3-3 protein was first identified by library screening. It is of course possible that the anti *C. sativus* phloem protein-specific antiserum recognised a different epitope of the 14-3-3 protein than the specific antiserum used for the confirmation of localisation.

14-3-3 proteins have been shown to play diverse roles in many biological processes in plants. They were shown to regulate key plant metabolic enzymes (Bachmann et al., 1996; Toroser et al., 1998; Moorhead et al., 1999) including also lipoxygenase (Holtman et al., 2000) an enzyme that was previously found in the phloem (Avdiushko et al., 1997). However, the best understood interaction between 14-3-3 and a target protein is the activation of plasma membrane H^+ ATPases (Alsterfjord et al., 2004). ATPases play a crucial role in plant cells by generating a proton gradient thus providing the driving force for e.g. phloem loading. One H^+ ATPases was shown to be expressed exclusively in CC (DeWitt and Sussman, 1995) and also vascular localisation of one of the tobacco 14-3-3 proteins has been previously demonstrated (Moriuchi et al., 2004). Additionally it was demonstrated by Pnueli *et al.* (2001) that 14-3-3 proteins can potentially interact with FT (flowering locus T), a protein that has been recently found in phloem sap of *B. napus* (Giavalisco et al., 2005) and which is thought to function as a flowering induction signal. It is tempting to speculate that 14-3-3 together with FT proteins are involved in long-distance signalling for flower induction in the phloem.

Another interesting cDNA identified from the *B. napus* library is coding for a double-stranded DNA binding protein (Table 1). Several proteins that can bind nucleic acids have already been described to occur in SE {Gómez, 2004 #163;Gomez, 2005 #162;Xoconostle-Cazares, 1999 #48}. These were, however, mostly RNA binding proteins (discussed in the following section). The evidence only exists that single-stranded nucleic acids are transported by proteins, but it can not be excluded that double stranded forms of RNA or DNA are transported as well, see for review (Carrington et al., 1996).

Another protein fished from *B. napus* library was identified as RIO1, a serine-specific protein kinase containing an ATP binding motif (Angermayr and Bandlow, 2002). Several protein kinases were identified in the SE exudates from C. maxima (Yoo et al., 2002) and were speculated to be involved in the signal transduction within the phloem. Additionally, Nakamura *et al.* (1993) found in the phloem sap of rice, kinase

activities that were Ca^{2+} dependent. It can be speculated that RIO1 can also function in the phloem as a protein kinase being involved in the long-distance signalling.

A protein with homology to *A. thaliana* cytochrome b5 domain was found in *C. sativus* phloem sap. Cytb5 takes part in lipid biosynthesis in plants was found in microsomes of numerous species including *Brassica* (Kearns et al., 1991). Membrane bound fatty acid desaturases require cytochrome b5 together with Cb5R as an electron donor for activation (Kearns et al., 1991). Concerning the information on Cb5 from animals and yeast it is speculated that cytochrome b5 can operate as a direct or indirect electron donor in the biosynthesis of products such as sterols, terpenoids and signalling molecules like gibberellic acid. A Cb5R enzyme was previously found in the phloem and it was hypothesised that Cb5 together with Cb5R take part in Fe signalling between shoots and roots (Grusak and Pezeshgi, 1996; Xoconostle-Cazares et al., 2000). The phloem Cb5R from pumpkin was described to have the capacity to interact with plasmodesmata increasing their SEL and to potentiate its own cell-to-cell transport (Xoconostle-Cazares et al., 2000).

For several of the cloned cDNAs no functions in the phloem could be assumed. One of these proteins is plastocyanin, a chloroplast protein and member of the photosynthetic electron transport machinery, which is not known to have any alternative function in plants (Redinbo et al., 1994). Since mature SE do not contain chloroplasts, the detection of photosynthetic proteins may be an effect of contamination from neighbouring cells during phloem sampling or they may be remnants from immature SE. The first suggestion of a contamination seems rather unlikely, because, as described above, sample purity seems to be very high. It can also not be excluded that such proteins can have alternative function in the phloem that has not yet been described. For proteins like transducine (WD-40 protein) that belongs to a group of ancient regulatory proteins found in all eukaryotes, it is also difficult to predict a specific function in the phloem because of the very wide functional spectra of this protein group. They regulate cellular functions such as cell division, cell-fate determination, gene transcription, transmembrane signalling, and mRNA modification (Neer et al., 1994). The same holds true for the esterase identified from the *C. sativus* library. On the basis of its partial cDNA sequence obtained from the library clone, the cloned esterase belongs to a big family of different plant esterases that can have many functions in plants.

4.2.2 Identification of *B. napus* phloem sap proteins using 2 DE separation followed by ESI-MS/MS analyses

Different mass spectrometric methods like matrix-assisted laser desorption ionisation coupled with post source decay MS (MALDI-PSD), matrix-assisted laser desorption ionisation coupled with quadrupole time-of-flight MS (MALDI-Q-TOF) or ESI-Q-TOF MS have successfully been used for phloem protein analyses within the last years (Kehr et al., 1999; Haebel and Kehr, 2001; Walz et al., 2002; Barnes et al., 2004; Walz et al., 2004). The majority of these studies were performed on cucurbits and *R. communis*. In this thesis, phloem proteins from *B. napus* were separated by 2 DE and analysed by ESI-Q-TOF MS/MS. From the obtained partial amino acid sequences and their comparison with database entries, several proteins could be identified. The presented results were obtained in the framework of a large project aiming at a comprehensive overview about the rape phloem proteome, where proteins from 2 DE were additionally analysed by MALDI-TOF MS. The identification of *B. napus* phloem proteins from their partial amino acid sequences had a relatively high success rate (Giavalisco et al., 2005) in comparison to the analysis performed in other plant species like cucurbits and *Ricinus* (Barnes et al., 2004; Walz et al., 2004). This is due to the fact that *B. napus* genes share almost 90% sequence identity with their *Arabidopsis* homologues (Cavell et al., 1998). These results stress the suitability of *B. napus* as a model plant for phloem protein analyses.

As already discussed in the previous section, stress response and defence related proteins are an abundant group of polypeptides in phloem sap (Walz et al., 2004). Several members of the myrosinase defence system were identified in this work (Fig. 15). This defence system is characteristic for the *Brassicaceae* family and consists of glucosinolates and different classes of myrosinases (thiglucosidases) (Lenman et al., 1990; Rask et al., 2000). Thioglucosidases in response to tissue damage, caused for example by herbivory, degrade glucosinolates to produce toxic nitriles, isothiocyanates and thiocyanates. The presence of myrosinases in the phloem of *B. napus* was confirmed additionally by western blot analysis (Fig. 16). Myrosinase-specific antibodies (Lenman et al., 1990) detected a single band in the size of approximately 70 kDa in phloem samples of *B. napus*, and this observed size of the protein is consistent with the known *B. napus* myrosinases detected previously with the same antibodies (Lenman et al., 1990). The signal was absent from the phloem of cucurbits, which was expected since cucurbits do not contain myrosinases. There

was also no cross-reacting protein in rape leaf extracts, what might be caused by the low level of myrosinase in comparison to the total protein content. From the obtained partial amino acid sequences it was not possible to assign the identified phloem myrosinase to any particular myrosinase class (Fig. 22). Myrosinases were highly abundant in phloem sap samples of *B. napus* (Fig. 15). Moreover, two myrosinase binding proteins (Fig. 15) were identified in analogue samples by Giavalisco *et al.* (2005). Upon wounding, in example by herbivores, myrosinases have been shown to form complexes with myrosinase binding proteins (MBPs) this process however seems not to have influence on the catalytic myrosinase activity (Eriksson et al., 2002) and the functions of MBPs remained so far unclear. In addition to the proteins found in the phloem sap of *B. napus*, glucosinalates have been detected in the phloem exudates of *A. thaliana* and there are strong indications that they are transported *in vivo* (Chen et al., 2001). The co-existence of glucosinolates and the myrosinase system in one compartment is surprising but seems possible if the system is tightly regulated, e.g. by post-translational modifications of the involved proteins. Myrosinase proteins, as can be seen in Fig. 15, seem to be highly modified indicated by a number several distinct protein spots. Alternatively, myrosinases could also be stored in the plastids of undisturbed SEs and released into the glucosinolate containing sap by the disruption of plastids during mechanical damage (Knoblauch and van Bel, 1998). In both examples, such a myrosinase system would constitute a potent line of defence against insects or pathogens invading the phloem by allowing an instantaneous up-regulation of glucosinolate cleavage into toxic compounds.

It is almost certain that many proteins and RNAs traffic simplasmically over short and long distances to regulate fundamental plant processes (Ding et al., 2003). Different forms of RNA have been shown to be translocated including endogenous mRNAs (Sasaki et al., 1998; Ruiz-Medrano et al., 1999; Xoconostle-Cazares et al., 1999; Kim et al., 2001), pathogenic RNAs {Gómez, 2004 #163;Carrington, 1996 #164} and small RNAs related to gene silencing (Palauqui et al., 1997; Mlotshwa et al., 2002). RNA is therefore speculated to be a long-distance information molecule in plants (Lucas et al., 2001; Yoo et al., 2004). It seems likely that RNAs are transported as ribonucleoprotein (RNP) complexes analogically to RNP complexes formed by viral RNAs with their corresponding movement proteins (Thompson and Schulz, 1999; Lucas et al., 2001). So far, several phloem proteins, e.g. CmPP16 (Xoconostle-Cazares et al., 1999) or cucurbit PP2 {Gómez, 2004 #163} were demonstrated to

have RNA binding properties and to translocate RNA within the phloem. In this work, proteins from two distinct classes of RNA binding proteins were identified in the phloem using two separate approaches. One of them, the 17 kDa phloem lectin, was found in the phloem of cucumber by expression library screening and was discussed in the previous section. The other RNA binding proteins belong to the glycine-rich RNA binding protein (GRP) group and were detected in the phloem of *B. napus* by MS/MS (Fig. 15). The partial amino acid sequences obtained for the *B. napus* phloem GRP showed the highest homology to *B. napus* GRP 10 and to GRP 8 and 7 from *A. thaliana* (Fig. 21). Recently, also Barnes *et al.* (2004) identified a glycine-rich RNA binding protein in SE exudates from *R. communis*. The presence of the putative GRP 7 in the phloem sap was verified by western blots using peptide antibodies (Fig. 16). The size of the single band marked with antibodies (approximately 14 kDa) appeared to be slightly different from the known GRP 7 protein from *A. thaliana* which is approx. 17 kDa big (Nocker and Vierstra, 1993). However, the so far described GRPs from *Arabidopsis* are nuclear proteins and phloem-specific GRPs should be rather cytosolic proteins and therefore could differ in size. It has been demonstrated that mRNA levels of some members of the GRP family are elevated upon low temperatures and decreased by ABA treatment (Sturm, 1992; Carpenter et al., 1994; Nomata et al., 2004). A possible function for phloem GRPs would therefore be related to long-distance RNA transport modulating responses to stress conditions.

Besides the possible RNA signalling phenomenon, it is very likely that signalling involving Ca^{2+} as a second messenger exists in the phloem. It is thought that sudden elevation of the normally low level of calcium inside SE that can occur upon stress and/or SE disruption, can cause a signalling cascade (Knoblauch et al., 2001; van Bel and Gaupels, 2004). Several calcium binding regulatory proteins that belong to calmodulins have been reported to be present in the phloem (Barnes et al., 2004; Giavalisco et al., 2005). Another group of proteins that can possibly bind Ca^{2+} ions are annexins, detected in this study in the phloem of *B. napus* (Fig. 15). From partial amino acid sequence comparisons annexin 1 and annexin 2 have been identified in the phloem samples (Fig. 20). Annexins are known as cytoplasmic Ca^{2+} binding regulatory proteins with the ability to interact with membranes in a calcium and phospholipid dependent manner (Andrawis et al., 1993; Clark and Roux, 1995). Recently annexin 1 and 4 from *A. thaliana* have been shown to mediate osmotic stress and ABA signal transduction, but the functions of the other six annexins

remains unclear (Lee et al., 2004). However, a different function of the phloem annexins can also be assumed on the basis of recent studies on cotton annexins. Andrawis *et al.* (1993) showed that callose synthase is regulated by cotton annexin in a calcium dependent manner. Formation of callose participates in the closure of SE upon injury and annexins could possibly play a mediating role in such a process.

The capability of phloem proteins for functionally binding calcium ions could be confirmed in additional experiments where calcium-binding proteins were isolated.

It was shown in the experiment with immobilised Ca^{2+} ions that several phloem sap proteins can bind calcium ions (Fig. 18) however their identity could not be confirmed due to the very low amounts of protein eluted from the column. Probably the efficiency of protein binding to immobilised Ca^{2+} was too low.

4.3 Selection of Arabidopsis T-DNA insertion lines

To study the possible functions of some of the phloem proteins, different knock-out mutants containing T-DNA insertions within the genes encoding these phloem proteins were investigated. It was assumed that the high homology between the genomes of *B. napus* and *A. thaliana* would enable using the available mutant collections from *A. thaliana.* This assumption was supported by immunolocalisation studies where anti *B. napus* phloem protein-specific antiserum (Fig. 19) was used. These experiments demonstrated that *B. napus* phloem-specific antibodies localise also to the phloem of *A. thaliana*. This indicates a high similarity between the phloem proteins of both species.

Candidate proteins were selected from the pool of identified phloem proteins due to the interesting possible roles they can play in the SE sap as discussed above and on the basis of sequence homology between proteins identified in *B. napus* phloem sap and known proteins from *A. thaliana*. Available mutants for GRP 7, myrosinase and annexin 1 and 2 were employed for further studies.

For all three KO mutants, homozygous lines could be obtained. Different lines from every mutant were compared to the wild type Col-0 being in the same developmental stage and growing under identical, normal conditions in order to estimate an obvious phenotype. Although no drastic phenotype was observed for any of the mutants (Fig. 24), it can not be excluded that the investigated mutants could show changes in growth and development under stress conditions. In fact every of the studied proteins

has been suggested to be involved in different kinds of stress responses, as discussed in the previous sections. Therefore, further phenotypic studies under different growth conditions might allow gathering more knowledge about the possible functions of the investigated proteins in the phloem.

4.4 Grafting experiments to study the transport of phloem sap proteins

Grafting experiments where wild type plant served as the stock and KO lines as the scion were employed to investigate translocation of the studied proteins. These analyses were performed on the GRP 7 mutant as the GRP protein seemed to be very interesting due to its RNA-binding properties as discussed above. Grafting experiments were performed successfully (Fig. 25). However, the detection of GRP 7 protein in the WT and KO lines using anti GRP antiserum did not allow discrimination between these plants. The antiserum used for western blot seemed to react with more than one protein in the phloem of *Arabidopsis* and no differences in the protein pattern between WT and mutant stem extracts could be observed (Fig. 26). Concerning the fact that the antiserum was produced against a 15 aa peptide it can not be excluded that it is able to recognise other GRP proteins or proteins different than GRP but with similarity to this 15 amino acid sequence. Sequence similarity searches with the 15aa peptide used for antibody production, however, did not result in an identification of any other proteins highly similar to GRPs (data not shown).

5 Summary

The major aim of this work was the identification of new phloem sap proteins and a metabolic characterisation of this transport fluid. The experiments were performed on the three plant species *C. sativus*, *C. maxima* and *B. napus*.

To characterise the phloem samples from *B. napus*, a new model plant for phloem analysis, western blot tests together with metabolite profiling were performed. GC-MS metabolite profiling and enzyme assays were used for measuring metabolites in the phloem of *B. napus*. Results from the phloem sap measurements showed, as expected, a typical sugar distribution for apoplasmic phloem loaders with sucrose being the predominant sugar. In stem extracts, the most abundant sugar was glucose with much lower fructose and sucrose levels. With the GC-MS approach it was possible to identify a number of metabolites which showed a differential distribution when phloem and stem tissue extracts were compared.

For protein identification, two different approaches were employed (i) screening expression libraries with total phloem protein specific antisera and (ii) protein separation on 2 DE gels followed by ESI-MS/MS sequence analyses. For the first approach, three different phloem protein-specific antisera were produced and expression libraries were constructed. Phloem protein antisera were tested for specificity and some attempts to estimate specific epitopes were undertaken. Screening of the libraries resulted in the identification of 14 different proteins from all investigated species. Analyses of *B. napus* phloem sap proteins from 2 DE with ESI-MS/MS resulted in the identification of 5 different proteins. The phloem localisation of the identified proteins was additionally confirmed by western blot tests using specific antibodies.

In order to functionally characterise some selected phloem proteins from *B. napus,* the group of potential calcium-binding polypeptides was analysed for functional Ca^{+2} binding properties and several Ca^{+2}–binding proteins could be isolated. However, their sequences could as yet not be determined.

Another approach used for functional protein characterisation was the analysis of *Arabidopsis* T-DNA insertion mutants. Four available mutants with insertions in phloem protein-specific genes were chosen from the SALK and GABI-Kat collections and selected homozygous lines were tested for the presence of the investigated

proteins. In order to verify if the product of one of the mutated gene (GRP 7) is transported through the phloem, grafting experiments were performed followed by western blot analyses. Although the employed antiserum against GRP 7 protein did not allow distinguishing between the mutant and the wild type plants, successful *Arabidopsis* grafting could be established as a promising method for further studies on protein translocation through the phloem.

6 Deutshe Zusammenfassung

Das Hauptziel der vorliegenden Arbeit war die Identifizierung neuer Phloemsaftproteine sowie die metabolische Charakterisierung dieser Transportflüssigkeit. Die beschriebenen Experimente wurden an den vier Pflanzenarten *Cucumis sativus*, *Curcurbita maxima*, *Brassica napus* und *Arabidopsis thaliana* durchgeführt.

Für die Analyse von Phloemsaftproteinen aus *B. napus* war zunächst wichtig, die Herkunft und Reinheit der gewonnenen Phloemproben mittels Western Blot Analysen, Bestimmungen der Zuckerkonzentration und einer metabolischen Charakterisierung mittels GC-MS zu überprüfen. Die Ergebnisse dieser Untersuchungen zeigten erwartungsgemäß die typische Zuckerzusammensetzung für apoplastische Phloembelader, mit Saccharose als vorherrschendem Zucker. In Stängelextrakten war dagegen Glukose der vorherrschende Zucker, wogegen Fruktose und Saccharose in wesentlich geringeren Mengen nachweisbar waren. Mit diesem GS-MS Ansatz war es möglich eine Vielzahl von Metaboliten zu identifizieren, die eine differentielle Verteilung in Phloemsaft und Stängelextrakt aufweisen.

Ein Versuchsansatz zur Proteinanalyse war es Gene, welche Phloemproteine kodieren, zu klonieren. Zu diesem Zweck wurden Expressionsbibliotheken von *C. maxima*, *C. sativus* und *B. napus* konstruiert und anschließend mittels Phloemprotein-spezifischen Antiseren der entsprechenden Arten durchforstet. Des Weiteren wurden die Antiseren auf ihre Spezifität getestet und es wurden Versuche durchgeführt, um mögliche spezifische Epitope zu identifizieren. Die Durchforstung der Expressionsbibliotheken führte zur Identifizierung von 14 verschiedenen Proteinen aus den drei untersuchten Pflanzenarten.

Im Rahmen einer umfassenden Charakterisierung des Phloemproteoms von *B. napus* mittels zwei-dimensionaler Gelelektorphorese (2 DE) und anschließender Massenspektrometrie (ESI-MS/MS), wurden insgesamt 5 neue Phloemproteine identifiziert. Die Lokalisierung dieser Proteine im Phloem wurde zusätzlich mittels Western Blot Analysen unter Verwendung spezifischer Antikörper verifiziert.

Die funktionelle Analyse einiger dieser neu identifizierten Proteine wurde unter Zuhilfenahme von „knock-out“ Mutanten der Modelpflanze *Arabidopsis thaliana*

begonnen. Das vorrangige Ziel dabei war, zu überprüfen, ob die ausgewählten Proteine tatsächlich im Phloem transportiert werden. Dazu wurden so genannte Pfropfungsversuche durchgeführt. Vier *Arabidopsis* Mutanten, die Insertionen in Phloemprotein-spezifischen Genen aufweisen wurden aus der SALK bzw. aus der GABI-KAT Sammlung ausgewählt und die selektierten, homozygoten Linien auf die Anwesenheit der entsprechenden Proteine hin untersucht. Um letztendlich den Transport eines der mutierten Genprodukte zu verifizieren (GRP 7), wurden Pfropfungsversuche mit anschließender Western Blot Analyse durchgeführt. Obwohl das entwickelte Antiserum gegen GRP 7 es nicht erlaubte zwischen mutierten und Wildtyp Pflanzen zu unterscheiden, konnte doch die Pfropfung von Arabidopsis Pflanzen erfolgreich etabliert werden. Somit steht nun eine Erfolg versprechende Methode zur Verfügung, um den Proteintransport durch das Phloem zu untersuchen.
Eine weitere Gruppe von Phloemproteinen aus *B. napus*, solche mit einer potentiellen Calcium-bindenden Domäne, wurden ebenfalls funktionell auf mögliche Ca^{+2} -bindende Eigenschaften hin untersucht, dabei konnten einige Ca^{+2} –bindende Proteine isoliert werden, deren Sequenz bislang jedoch noch unbekannt ist.

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8 Appendix

	phloem [Mol]			**stem** [µmol/g FW]		
	average		SD	average		SD
glucose	0,015	±	0,002	190,732	±	14,797
fructose	0,001	±	0,001	42,411	±	8,854
sucrose	0,602	±	0,017	16,787	±	6,535

Table I Soluble sugars concentration measured enzymatically in phloem sap and inflorescence stem. Average values and standard deviations (SD) were calculated for n=8 measurements (phloem) and n=14 (stem).

mutant	**gene**	**LP**	**RP**	**T-DNA primer**	**annealing**
GRP	At2g21660	ttggctatatgtggctttgtt	agggtggaggaggatacagcg	tggttcacgtagtgggccatcg	62℃
Myrosinase	At5g26000	ttgacattcttctccctacaaaca	tggactcacgcacaactctc	cccatttggacgtgaatgtagacac	64℃
Annexin1	At1g35720	tggactcttgaacccggtgag	ccacactggttctctctgtctctc	gcgtggaccgcttgctgcaact	62℃
Annexin2	At5g65020	gtatggaaacgccattaacaag	tgggtgttatggagaagaaacttcg	tggttcacgtagtgggccatcg	63℃

Table II Nucleotide sequences of primers used for homozygous lines selection from A. thaliana T-DNA mutants; LP- left primer, RP-right primer, annealing- annealing temperature used in PCR reaction;

GRP	spot No.	**Myrosinase**	spot No.	**Annexin1**	spot No.
DAIEEFNGKELDGR	8	DFNFGVASAAYQVEGGR	1	HYSDEDVIR	5
GGGGGGYGGGG	8	YSNPLIYITENGFST	2	PSEDAEQLK	5
GYGGGGGGYGR	8	DFNFGVASAAYQVEGGR	2	TAHHTTGDFR	5
FSQFGEVIDSK	8	ENEPFTCNQTDQLSSK	2	DALLANEATKR	5
		DFNFGVASAAYQVEGGR	3	YQDEHGEEILK	5
		GGSDLGNGDTTCESYT	3	TLDKELTSDFER	5
		DFNFGVASAAYQVEGGR	4	VPSPSEDAEQLK	5
		GGSDLGNGDTTCESYT	4	YEGDEVNMTLAEQTAK	5
		YNNPLIYITENGFSTPGK	4	YEGDEVNFTLATQEAK	5
				SLEEGDEDDKFLGLLR	5

Annexin2	spot No.	**Thioredoxin h1**	spot No.
SAYAAAYNEDLLK	7	VVGAAKEEIEAK	12
ALDKELSSDFER	7	LDKVVGAAKEEIEAK	12
SIEEDVAQHTSGDLR	7	APIFVELAK	12

Table III Amino acid sequences of phloem protein spots from 2DE identified with Q-TOF MS-MS (according to the Fig. 15); GRP- glycine-rich RNA-binding protein;

	phloem			stem			phloem/stem
	average value		SD	average value		SD	
alanine	33858,84	±	6354,83	10891,18	±	3315,71	3,11
arginine	5208,48	±	1184,05	4265,80	±	913,22	1,22
arginine/ornithine	24804,57	±	3323,33	19703,04	±	2190,70	1,26
asparagine	2491,11	±	227,71	3831,88	±	264,51	0,65
aspartic	22573,54	±	1094,69	61352,02	±	2280,16	0,37
b-alanine	409,44	±	41,11	111,04	±	13,71	3,69
cysteine	665,16	±	130,59	305,51	±	23,78	2,18
dopamine	8,75	±	3,35	nd	±	nd	
g-aminobutyric	593,29	±	56,92	564,16	±	49,38	1,05
glutamic	36892,43	±	1405,15	58676,01	±	1238,39	0,63
glutamine	82652,23	±	7941,70	69187,41	±	1008,67	1,19
glycine	6351,11	±	590,77	13073,77	±	1912,40	0,49
homocysteine	2,20	±	0,46	nd		nd	
isoleucine	53779,89	±	2958,13	44165,75	±	1937,16	1,22
leucine	43577,31	±	1199,25	26013,59	±	2433,11	1,68
lysine	39254,95	±	2444,81	nd	±	nd	
methionine	21300,81	±	1968,52	1770,18	±	180,84	12,03
O-acet-serine	130,49	±	34,81	nd	±	nd	
phenylalanine	27786,67	±	1316,71	3264,30	±	178,49	8,51
proline	65811,16	±	15170,22	78619,80	±	1612,90	0,84
putrescine	26840,66	±	870,32	861,67	±	57,62	31,15
pyroglutamic-oxoproline	63909,53	±	22532,61	105174,84	±	6109,19	0,61
serine	58043,05	±	2223,44	62230,96	±	1149,11	0,93
S-me-cysteine	78,02	±	10,88	114,37	±	27,47	0,68
spermidine	553,54	±	874,32	nd	±	nd	
threonine	36031,25	±	7935,87	65803,30	±	1783,96	0,55
tryptophan	7428,24	±	3029,43	237,09	±	90,15	31,33
tyramine	nd	±	nd	365,87	±	35,33	
tyrosine	4903,89	±	469,28	408,52	±	38,72	12,00
uracil	53,35	±	14,73	53,56	±	4,05	1,00
valine	76730,86	±	2360,80	63141,58	±	1485,03	1,22
6-Pgluconate	14,43	±	2,63	nd	±	nd	
a-kt-glutarate	nd	±	nd	59,61	±	2,63	
citrate	53501,58	±	2016,80	42400,36	±	1990,77	1,26
dehydroascorbate	13904,67	±	4018,52	17941,66	±	4151,26	0,77
D-isoascorbate	36994,80	±	10088,93	12802,86	±	3146,58	2,89
fumarate	307,48	±	31,72	nd	±	nd	
gluconate	29,29	±	2,80	nd	±	nd	
glutarate	4,88	±	1,24	nd	±	nd	
glycerate	374,61	±	58,10	296,62	±	26,17	1,26
glycerate-3-P	1592,15	±	330,89	nd	±	nd	
glycolate	27,66	±	5,74	35,54	±	4,97	0,78
isocitrate	1417,90	±	175,82	812,96	±	101,90	1,74
L-ascorbate	7373,56	±	2821,37	43007,00	±	7809,04	0,17
maleate	17647,89	±	1503,80	521,44	±	81,32	33,84
malate	8889,45	±	791,81	48714,24	±	1663,44	0,18
phosphate	58271,05	±	1933,61	102624,93	±	4881,38	0,57
quinate	5732,51	±	504,65	2243,12	±	132,78	2,56
saccharate	29,29	±	2,80	455,15	±	69,94	0,06
shikimate	9795,08	±	2179,71	nd	±	nd	
succinate	17915,81	±	1551,24	7205,81	±	479,34	2,49
threonate	74,16	±	10,80	670,60	±	51,61	0,11
arabinose	153,82	±	10,53	76,85	±	2,73	2,00
erythritol	33,89	±	7,08	35,86	±	4,63	0,95
fru-6-P	2248,29	±	383,07	61,35	±	8,57	36,64
fucose/rhamnose	188,67	±	61,79	nd	±	nd	
galactose	135,35	±	6,12	115,35	±	5,66	1,17
gentiobiose	nd	±	nd	103,47	±	12,01	
glc6-P	8106,14	±	1357,80	115,76	±	18,53	70,03
glycerol-1-P	639,29	±	129,21	nd	±	nd	
maltitol	nd	±	nd	107,47	±	11,09	
maltose	224,45	±	26,91	55,12	±	7,22	4,07
mannitol	805,15	±	52,97	485,94	±	35,67	1,66
mannose	40,81	±	11,71	186,85	±	12,59	0,22
myo-ino	7092,74	±	584,85	23198,36	±	895,05	0,31
myo-ino-1-P	296,41	±	43,37	nd	±	nd	
myo-ino-2-P	300,75	±	46,59	25,03	±	2,97	12,02
raffinose	nd	±	nd	30,17	±	1,59	
rhamnose	356,23	±	104,32	nd	±	nd	
ribose	34,17	±	3,44	23,13	±	3,30	1,48
sorbitol/galactitol	805,15	±	52,97	53,05	±	15,97	15,18
trehalose	1133,69	±	182,61	126,01	±	8,92	9,00
xylose	333,17	±	18,56	38,05	±	2,06	8,76

Table IV List of metabolites measured on GC-MS in *B. napus* phloem sap and inflorescence stem. Standard deviation (SD) calculated for n=10 measurements (phloem) and n=5 (stem); nd- not detected;

clone name	Homology	Nucleotide sequence
C16	14-3-3	GGGATCAAAGGACGATTTTACTCAGCTCAGTTCTCTCTTCCTCCCGCACTA AGTCTCTCTAATGGCGTCCCTCATCCCTGAAAACCTAACAAGGGAGCAGTATGTTTACCT ATCCAAGCTTTCCGAGCAAGCCGAGCGCTACGAGGAAATGGTTGACTTCATGCAAAAGCT TGTATTGACCTCCACTCCTGCCTCCGAGCTCACTGTCGAGGAGCGCAATCTCCTTTCCGT TGCATACAAGAACGTGATCGGTTCTCTCCGAGCAGCTTGGCGCATCGTATCCTCCATTGA ACAGAAGGAAGAAGGAAGGAAGAACGAAGATCATGTCGTCCTCGTCAAAGATTACAGATC CAAGGTCGAATCCGAACTCACCGAGGTCTGCGCGAATATTTTGAAGCTTTTGGACTCCAA TCTCATACCATCTTCGTCCTCTGGTGAATCTGAGGTGTTCTACTTAAAGATGAAGGGAGA TTACCATAGGTATTTGGCTGAGTTTAAGATTGGGGAGGAGAGGAAGTCCGCCGCCGAGGA TACCATGCTAGCTTACAAGGCTGCTCAGGATATTGCAGTTGCCGATCTTGCACCAACGCA TCCNATCAGGCTGGGCCTAGCACTAAATTTCTCTGTTTTTACTTTGAGATTCTGAACCAG TCTGATAAAGCTTGTAGCATGGCCAAACAGGCATTTGAAAAAGCGATTGCGGAATTGGAC ACATTAGGTGAGGAATCATACAAGGTCTCTTAATTCATGTAGGATCTCATTTGGCTCAAA CCACTTGTAAGTAAAGTAAGTAAGACATTTCTAATGTTCTCTTAATTGGCAAAAAGTTGT ATAATTGAATGCTTTTTATATTATTAAAAAAAAAAAAAAA
C65-1	Rubisco activase 1	TCAGAAGCAAAGGCGGGCTGCTAAAGCTCAGATAAAGA GAGACGCCCATGGCAAGATCCCTAAACGTGGAGCCCAATAGCTTTACCGATCAGGTAGGG GAGCGACCAACATTATTCACAAGGTTTAATTTCTATCCACCATGTTCGACGCCACATCTT GTTCTTGGACTCAAAGAACACTCCGATGGTTCAGCTATCACCATTCTTCTACTCGACAAA CAAGTTGAAGGCCTCCAATTGCGAAAGGATGATCAGTGGTATAGAGTCCCTGTTCCTGCC ATTGCTGATTCTCTTCTCCTCGTCATTGGGGAACAAGCCGAAGTTATGAGTAATGGAATC TTCAAGAGCTCTGTTCATCGGGCGGTGACGAATTCCCGGGATCAAATACTTACACACTCC CAAGTCCTCATGGCCGCCTCCGCTGCCTCTGTCGGAGTCGTTAACCATGCACCGCTAAGC TTGAAAGGCTCTGGATCCACAACTTCGGTTCCAAGTTCAGTCTTCTTTGGGAACAGTTTG AAGAAAGTGGTCAACTCCAGAGTTGTCAACCCGAAGCCTTCCTCGGGTAGCTTCAAGGTG ATGGCAGTGGAATCAACCTCAGACGAGAATCTCGAGAAGAAGACCAAAAAAATAGACAGA TGGGCCGGACTTGGAACCGATATATCAGATGACCAACAGGACATCACAAGAGGAAAGGGA ATGGTGGATTCTGTGTTTCAAGCTCCCATGCAAGCTGGAACTCATTATGCTGTTATGAGC TCTTATGAGTATCTTAGTCAAGGACGTAAATCGTATGATGGCATGGACAATGTATTGGGT GGTTTGTACATTGCTCCTGCTTTCATGGACAAATTGGTTGTTCACATCACCAAGAACTTC CTGTCCCTCCCAAACATCAAGGTTCCTCTGATTTTGGGTCTGTGGGGAGGCAAAGGACAA GGAAAATCCTTCCAATGTGAGCTTGTATTTGCCAAGATGGGAATCACCCCAATCATGATG AGTGCAGGAGAATTGGAAAGCGGGAACGCAGGAGAGCCAGCCAAGTTGATCCGACAACGG TACCGTGAGGCCGCTGACATAATCAAGAAGGGAAAAATGTCCTGCCTCTTCATCAATGAT CTGGATGCGGGTGCTGGTCGTCTTGGTGGTACCACTCAATACACAGTCAACAACCAAATG GTTAACGCCACACTCATGAACATCGCTGATAACCCCACCAATGTCCAACTCCCTGGAATG TACAACAAGGAGGAGAACCCTCGAGTTCCAATCATCGTCACTGGTAACGATTTCTCCACT CTCTACGCTCCTCTCATCCGTGACGGTCGTATGGAGAAATTCTACTGGGCACCCACTA
C41-1	17 kDa phloem lectin	GGGATCTCTCAAATACCATTTTAATGGCAGGCCAA AGCACACATTATTTGGCATTTCCAAGAGCTTCCACAATAACATGGGGTGATGACACTCGA TACTGGAGTTGGGCCACCGTGGATTTTTGCAGCTACGCAATTGAAGAAGCCCGACTTTTA CAAGTATCTTGGCTCGATTGTCGTTGGAGCATGGATGCATCTGATTTCAAACAAGATATT TGGTACAATGCAAGCGTTGAAGTAATGTTGACAAGCAACGCCTCTGGATGGAATGTTCCA CTACACCTTGAAATCGAGTTGCCAGATGGGAGTACGCAAAAGTCTCAAATAGTATTGGCA GGCAGACAACCAAATGTGTGGTTCAAGATTCCAATCGGTAAATTCATACTAAGCGGTTCT CTGACTAGCGGAACAATCCGATTCGGCTTCTACAACCATGAAGGGAATTGGAAAAGAGGC TTGAACATAAGAGCCCTTGCCATTCAAGCATAAAAATGAGTTTAAAATGATATGTTCAAG AATGAATATAAAGTAATAGAATAAAACCCTAAATAATAAGATCTTTTGCTATGTTACAAA AACTTTTAAGTTTAAATGTGTTATATTTCAGTTATGCAAGTAAATGTTGAATATAATGTA ACACAACATAAATAATATGACCTTTTGCATTGTTAAAAAAAAAAAA
C24	Cytochrome b5 domain containing	ATCTCATTCCGATCAATCCCCTCCAAGCTGAGCTAATGGAGCTTACACCTCTACA ACTGTCGGTCTACAATGGCACCGACCCAACAAAGCCCATCTATGTTGCTTTGAAGGGCCA AATCTACAACGTCACATCAGGTCGTTCTTTCTACGGCTCCGGTGGTCCTTACGCCATGTT CGCCGGCAAGGACGCGAGCAGAGCTCTGGCCAAGATGACCAAGAATGAGGAAGATATCAC

	protein	CTCTTCACTCGAAGGCCTCTCTGAGAAAGAGATCGGTGTTCTCAACGACTGGGAGAACAA ATTTCAAGCTAAGTACCCTATTGTTGGCCGTGTTGTTTCTTAAACATGCAAGTTTACGAG TTTCGCTGTGTGGGTGTTTGTTGACTCATCGTAGTTTTCAGAAGTATTAAATGAATAATT GTTCAACTCTGAATATGGGGTTGCTGAAATGACTGTAAAGTTTGTTGTGCTTGGTTTAAT AAGGGTCTTCAATGGCGGATTGGTATCAGTCTGAACTGCTTGGTGAAATAAGAACTTGCT TATTTATTTCAAAAAAAAAAAAA
C34	Esterase	ATCGACAATCTCCCATTATCAACCATCTCTTGATTCCA TCCCCAAGATTTTTCTTCCAACACTCTGAAAATGGACATCAAGCCAACCGAAATCACCTG CTCCAAGATGTTCGGAGGATACAACCGGAGATACCGCCATTACAGCCCAACTCTTGGCTG CTCCATGACCTTCTACATTTACTTCCCTCCATCTTCTCTCCCGTCCAATAAATTTCCAGT ACTCTACTGGCTCTCTGGCCTTACGTGTTCGGATGAAAATTTTATAATCAAAACGGGGGC ACAACGCACTGCTTCCAGTGAAGGTGTTGCTCTAATTGCACCTGATACATCTCCAAGAGG CCTGAATGTTGAAGGAGAGTCAGACAGTTGGGATTTTGGTGTCGGTGCTGGATTTTATCT CAATGCTACACAAGAGAAATGGAAACATTGGCGTATGTATGATTATGTTGTCAAGGAACT ACCTAAACTTCTGAGTGAGAACTTTCCACAACTTGATACATTGCGGGCATCTATATTTGG TCACTCCATGGGCACTTGTACAAGCTTCAGAGGCGTCGCAGGTTGCGTAAGATTTCCATC CCCAACAGTCAAGCATTGTCCCTTTTGAGCAGGGACCTTCGCGAGGCTCATCTGCAAGTC CTTTTAGGGAGCAGTGGGTACCGTACAAGCACCACTAACGTGCCTAGTGCAACTCATGAT CCGTTTTTATCATCACTTATTTTAAATTATCCTGCATCTGATGTTGAAGATATCTCAAAA TCCATGCTAACGAGTGCCGAAGATGTGTCTTCAGAGAACGTGGCACCATCACCAATTTGG AAATCAAGTTTTGATCCTTCTTTGAGCCAGGAAGAGCGGGAAAAAAGGATGAGACAGGCT ATTGGGAGAGCAGGCTTTATGCGAGATATGCTTTTCTCAACTCTCTTAGAGCACTAATGA AAGCATTGTGTTGCAAGAAGAATTTGAATGTAAAAATTTCAGAATAGTGAACGACAGAAG GGGAGCATTATGCTTGTTGGCGCTCGAAAGTCAGCCGAGCAGGACAAGGAGCCAGACCAT AACCACCATACAAGGAGAAACTTGAAGAGAAAGTAAGATGGTCAACACAGCCTAAGAATC TCCAATTAAATGGCAACTATTGGGACTGTGCCTACAGCTACTTCCAAGGAAATTATCAAT GCTTTTGTTTTTAAAACCTTCTCTTTTCATTATGACACAACACAACTAGGTTGAATTATC ATTCTGAAATTGAGACGCTGCAATTTATTATAGCTTGATAATGAATTGGAATTTGCTGTTT T
C16-1	not definied	ATTTACGAGGCCGAGCCTCTTCTTCTCTTCTTCATCTTATTCACTCTTCTC GGAGCCATCGGAGCCCTAGGAGACCGTGGAAAATTCATCGACGATCCTGAACCCGCCACT GGATTGGAACGCGCCGTCATTCCTCCCGGGAAGAGCTTCAGATCCGCCTTAGGTCTCAAA GAAGGAGGTCCGTTGTTCGGATTCACCAAGGCCAATGAGCTGTTCGTCGGAAGATTGGCG CAGCTAGGGTTTGCGTTCTCGTTGATCGGAGAAATTATTACTGGAAAGGGAGCTTTGGCG CAATTGAACATCGAGACGGGAGTGCCGATCAATGAGATTGAGCCTCTGGTTTTATTGAAT GTTGTGTTCTTCTTCATTGCGGCAGTGAATCCTGGAACTGGTAAATTCGTGACGGATGAA GAAGATGATGAATGAAGAGATGGATTGTGCTTTAATTTTTTGTCTGTACATGATGAAATT AGCAAAAAAAAAAAAAAAAAAGG
C17	not definied	GGGATCAAATATCAACTAAAGCTCTCACAAAAAA ATGACAATTGGTATTGGTGGAGGAATTGGCCTTGGTCTTCCTGGTCTCAATATAAAGATC GGCGGAGGCATCGGTCTTGGGAATAAAAGGCCGGGGGGGTGCAAGAGTCATGATCCATCA AAAAAATGGAGTGTATGTCCCAATGATCGCGTTGTAGAAATATATGCTAGGTTTGCAGTG GATGAGTACAACAGAAAACATGGACGTAATCTTGTGTTTCAATCAGTTTTAGAAGCTTGG GTTTATGTTTATCCTTGTGGCAAGAAAGAGTATAGTATTGAATTAGTAGTAAGAGAAGGG TGTGGGAATCATGTCCTCAAGTATCATGCTGTTGTGACTGAAACAGGCTGTGCTGCTCGC AGGAAGACTCTGGTGTCTTTCGACCAAATCGACGACTAAAAGAGAAGTTATATGCATGCA ATCAAAGTTTATACTTATGTGTGAGAAGTACACAATAATAAATCTTATATGATATTGTAC TGTTCTACTCTCTTTCACTATCTCTGTTCTAAAATGATAATGGAGGCCAAACTACGTTTT TAGTCCCTAACCCTA
C43-1	Unknown protein AC016661.7	TCCTTCGGGGCGATCTCCGGCCGGATCC CAAAATGGCGAACTCACCCTTCCGCCTCCCTTTCCTCCTCCTCCTTCTCGCCCTCCTAGC CTTCTCCTCCACCGCCGAGATCAAATCCCTCAAGATATCCTCCGACAACCGTCCCATGAT TCTCTTCGAGAAATTTGGTTTCACTCACACCGGTCAGGTCTCCATTTCCGTCAGTTCCGT CGCTGTTGCTACCTCCCTTGCTGAAACCGATCCTTCTCGTCTTGGATTCTTCCTCTTATC TGAAGAATCGCTTCTTCAGGTTCTTCTCGAGATCCAGCAAAACCCTCAGTTTTGCGTTCT TGATTCCCATTACATCCAACGTCTATTTACCTTCCGGGATCTCTCTCCCCCACCACAGAG CTCGTTTAGCCATTCTTATCCTGTTACTGCCCCCAATGAATACAGTCTTTTCTTCGCTAA TTGTGCCCCGGAAACATCGGTTTCTATGCAGGTTCGAACGGAGGTTTTCAATTTGGACCG CGATGGTTCCAAGGATTACCTCTCCGCTGGTCTTACTCAGCTCCCTTCGCTTTATTTTGT

		GTTTTCTCTTGCCTATCTTGCCTTCTTAGGGCTTTGGATCTATGCGGGTATCACGAATAA GCGAAGCGTTCACAGGATCCACTTGTTGATGGGTGGATTGTTGTTGATGAAAGCGTTGAA TCTCATTTGTGCTGCTGAGGATAAGCATTACGTCAAAAATACAGGAACGCCTCATGGTTG GGATGTGTTGTTTTACATCTTTCAGTTTATTCGGGTTGTTTTGCTTTTCACGGTGATTGT TTGNATCGGAACTGGATGGTCCTTTTTGAAGCCGTTTTTGCAAGAGAAGGAGAAGAAGGT GTTGATGATTGTGATCCCACTTCAAGTTTTGGCTAATGTTGCCTCGGTTGTGATCGGCGA GACTGGGCCATTTATTAAGGATTGGGTCACTTGGAATCAGGTTTTCTTGTTGGTGGATAT CATATGTTGCTGTGCCATAATTTTCCCCATTGTTTGGTCGATTCGATCTCTGAGAGAAAC ATCCAAAACCGATGGGAAGGCTGCAAGGAATTTGGCAAAGCTCACTCTTTTCAGGCAGTT CTACATTGTTGTGATTGGATATTTGTATTTTACACGAATTGTTGTTTTTGCACTCAAGAC CATTGCAGCATACAAGTATCAATGGGTGAGCAATGCAGCTGAGGAGATTGCAAGCCTTGT CTTTTACATGGTGATGTTCTATATGTTTAGGCCTGTTGAGAGAAATGAATACTTTGTTCT CGACGAGGAAGAAGAAGAAGCTGCAGAGTTGGCTCTGAGGGACGAAGAATTTGAGCTTTG AAATGGGTATTGAAGAGTATTTTTCTAGTTCTTATTGCCATATTTTGCTTCTGCCATACA AATGCAAGTGATATAGCTGATTTACAAAAAAAAAAAAAAAA
C60-1	not defined	ATCAAAATTTTCATTACTATCTTATATCAACTAAAGATCACAAAAAAA TGGCAGGTATTCTTGCTGGTCTTGATGGAGGACTTGGTTTTGGTTTTAGTCCTTTCAGTT TAAAGATCGGCGGAGGTATCGATCTCGGGAGTAAAAGGCCGGGGGGGTGCAAGAGTCATG ATCCATCAAAAAAATGGGTTATATGTCCCAATGATCAAGTTGTAGAAGCTGCTAAGTTTG CAGTGGCTGAGTTCAACAAAAAATATGGAAATAATCTTGTGTTTCAAGTAGTTTTGGAAG CTTGGGTTTTTACTAATCCTTGTGGGAAGAAAGAGTATAGTATTGAATTAGTAGTAATAG ACGGCTGCTTGAATCGTGTTCTCAAGTTTCATGCAGTCGTGACTGAAACACCCTGTGCTG CTCGCAAGAGGACTCTGGTGTCTTTCGACCAAATCGTCGACTAAAAGAGAAGTTATATGT GCAATCAAAGTTTATACTGTGTGAGAAGTACACAATAATAAATCTTGTAGGATATTGTAC TGCTCTACTTTCTTTCACTATCTGTTCTAAAATAATAATAATGGAGGCCAAACTACGTTT TTAGTC

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clone name	Homology	Nucleotide sequence
P17-1	dehydrin	GAGAGTTCTACTGCAGAACTTTGAAATGGGCTTGACGAGAGCCATCTCGGCGGAGGCAGC CATGGCGGATTACGACAACAAGAAGGTCGAGGAGGGTGTTCAAGAGGGTGGTGTCGAGGC CACGGATCGTGGGCTCTTTGATTTCTTGGGGAAGAAGAAAGAGGAAGAGCAGGCCGAGAA GCCCTCTGTTCATGAGGAAGAGGTGGTTGTAGTTACTGAGCAGATGGAGAAAGTTGAAGT TTCTGAACCTTCTCATAAGGTTGAACAAGAAGAAGAAGAGAAGAAGCCTAGTCTCTTGGA GAAACTCACCCGATCCGATAGCAGCTCTAGCTCTTCTAGCGATGAGGAGGAAGGAGAGGA CGGAGAGAAGAAAAAGAAGAAGAAGAAGGGATTGAAAGAGAAGTTGAAGGAGAAGCTAGG AGGAGGAGAAGAGGAAAAGAAGGAAGAAGAAGCTAAAAAGCACGAGCACGAGCACGAACA CGAAGCAGTGCCCATCCCAGTGGAGAAGGTGGAGGAGGCAGCACATCCAGAGGAAAAGAA AGGGTTCCTAGACAAGATCAAGGAGAAGCTTCCAGGCCACAGCAAGAAGCCAGAGGAAGC TCCAGAAGCGCCGGCTCCGTGCGCCACCGAGGCTGCAGCTCCTCCTCATCATCATGAAGA AGATCAAGGAAAAGAAAAGAAGGGATTTTTGGAGAAAATAAAGGAGAAACTCCCAGGGTA TCACGCTAAGGAGGATCAAGAGAAGCACAAGGAGGAGGCAGCTTCTCATTGAAGATGATG ATGATATGATATAATAGTCCATAATGGTTTAGATCAAGATCATAAATCATCATCATTGTG GCGTGGAGTTCTTGATTATTAATAATGCTGTTTTTTATTTAATTATGTGTTTTTGTAAGA ATTATATATGTGCTTTTTTGATGTGCATATATACATATGGGATTTGCTTTAAGGTGGGGG AGGGGGTTGTGTTTTCTTTTTCAATGTCTTTTCATTATATAACAGTGTTCATTTTGTATT TAGTTCTTGTGTGTGTTTTATAAAGCTATAAAAGAAAAAAAAAAAGTGTTCACTTAATGAA
P2	LHS1	GAGAGTTCTACTGCAGAACTTTGAAATGGGCTTGACGAGAGCCATCTCGGCGGAGGCAGC CATGGCGGATTACGACAACAAGAAGGTCGAGGAGGGTGTTCAAGAGGGTGGTGTCGAGGC CACGGATCGTGGGCTCTTTGATTTCTTGGGGAAGAAGAAAGAGGAAGAGCAGGCCGAGAA GCCCTCTGTTCATGAGGAAGAGGTGGTTGTAGTTACTGAGCAGATGGAGAAAGTTGAAGT TTCTGAACCTTCTCATAAGGTTGAACAAGAAGAAGAAGAGAAGAAGCCTAGTCTCTTGGA GAAACTCACCCGATCCGATAGCAGCTCTAGCTCTTCTAGCGATGAGGAGGAAGGAGAGGA CGGAGAGAAGAAAAAGAAGAAGAAGAAGGGATTGAAAGAGAAGTTGAAGGAGAAGCTAGG AGGAGGAGAAGAGGAAAAGAAGGAAGAAGAAGCTAAAAAGCACGAGCACGAGCACGAACA

		CGAAGCAGTGCCCATCCCAGTGGAGAAGGTGGAGGAGGCAGCACATCCAGAGGAAAAGAA AGGGTTCCTAGACAAGATCAAGGAGAAGCTTCCAGGCCACAGCAAGAAGCCAGAGGAAGC TCCAGAAGCGCCGGCTCCGTGCGCCACCGAGGCTGCAGCTCCTCCTCATCATCATGAAGA AGATCAAGGAAAAGAAAAGAAGGGATTTTTGGAGAAAATAAAGGAGAAACTCCCAGGGTA TCACGCTAAGGAGGATCAAGAGAAGCACAAGGAGGAGGCAGCTTCTCATTGAAGATGATG ATGATATGATATAATAGTCCATAATGGTTTAGATCAAGATCATAAATCATCATCATTGTG GCGTGGAGTTCTTGATTATTAATAATGCTGTTTTTTATTTAATTATGTGTTTTTGTAAGA ATTATATATGTGCTTTTTTGATGTGCATATATACATATGGGATTTGCTTTAAGGTGGGGG AGGGGGTTGTGTTTTCTTTTTCAATGTCTTTTCATTATATAACAGTGTTCATTTTGTATT TAGTTCTTGTGTGTGTTTTATAAAGCTATAAAAGAAAAAAAAAAGTGTTCACTTAATGAA CTTTGTTTTGTTTAAAAAAAAA
P14-1	Transcriptional coactivator-like protein	CTCACTAAGGGAACAAAAGCTGGAGCTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAA AGAATTCCCGGGATCCCAGGAACCCCTTTGTAACGAGAAACGGTAACACGAAGAAAGTTG AAATGGCTGGAATCGGACCACTTTCACAGGACTGGGAACCGGTTGTCATCAGGAAGAAGG CACCTAACGCCGCCGCAAAGAAGGATGAGAAGGCTGTTAATGCTGCACGCCGAGCCGGGG CGGAGATTGAAACCATAAAGAAATCTGCTGCTGGGTCGAACAAATCTGCCTCCAGCAGCA CTACTTTGAATACTAGGAAGCTTGACGAAGAGACGGAGAACCTTTCTCATGATCGTGTTC CAACTGAGCTGAAGAAAGCAATTATGCATGCTCGAACAGAGAAGAAGCTTACACAGTCTC AACTTGCTCAACTTATTAATGAGAAGCCTCAAGTTATCCAGGAGTACGAATCTGGAAAAG CTATTCCAAATCAACAAATAATAACCAAACTTGAGAGAGCTCTTGGAGCGAAATTGCGTG GGAAGAAATAAAGAGATATGGAAAGTTAAGTATGAGTTCTACGTAAGCTTTTGTTTGAAC CATGCTAAAAAATTCATACCCTACCGTGGTTTCAATGTGTAATTATGTTCGACCATCCTC GTATTCCTGTATAACCACTGTTGCAGTGTCTTGGTGAGTTCTTACCTGGAAATAAATAAA TATTGTGTCTGTCATGTTGCGTTAGTTTCCAGATGTTTTGGCTACACTTATTACATCAGA ACTTGGCTTTATTTGAATTTAATGGCTGCAGCCATGACAATTAACAATCATCAGAACTTG GCTTTATTTGAATTTAATGGCTGCAGCCATGACAATTAACAATCATCAGAACTTGGCTTT ATTTGAATTTAATGGCTGCAGCCATGACAATTAACAATTCAAAAAAAAAAAAAAAAAAA
P20-1	Transducine family protein	CAGCTCGAATTACCCTCATAAAGGGAACAAAAGCTGGAGCTCGCGCGCCTGCAGGTCGAC ACTAGTGGATCCAAAGAATTCCCGGGATCTTATTACTCCCTCTGAGATACTGATGGCTGT TTCTTCGTCTGAAACAACCAATATCATTGAAGGTGGTAAGAGTGATAGTGAGACAAATAT CCAGGATGTAGTGGTTAACAATGATAATGAAGATGCGGAGCTGGAGGTTAAAGAGGTGGG TGAAATGAAGTCTCCTCAGAATGGTGAATATGGTAGTAGAGGTGAGCCTCAAAACCTTTC TCTGGAGAACAAGGAAAAATATTTCTGCTCACAAGCGTCAGATCTTGGTATGGAAGTGGC CCGGGAGTGTAGTGCACTATCATCTGAAACTTATGTCATTGAGGAAGCCCCGCAAGTTGA TGGTAATATAATAGCATCAGAGGTTGATTCCCAAGCTGGTGAGGGAGATAGAACTTCCGG CAAAGATGTGTCTGATAAGCTTCCTGAATCATCTATGTCGACGACTTTGCAAATTCCAAC TCCTAGTTCAAAGGGGAAAAAGAACAAGGGGAAAAATTCTCAAGCTTCAGGCTTTGTTTC TCCATCTCCAAGTGCTTTCAATTCTAATGAATCGTCCATTGAACCTTGTGGTAGTTCAAC CCTTCCCCAAAGCGATGCAGCTTTCCCTCCTTTACTGGCCATCCAAGATACGTTGAATCA GATAATGAGCACTCAGAAAGAAATGCAAAAGCAGATGCAGATGACATTTTCAGTTCCAGT CACGAAAGAAGGTAAAAGGCTGGAGGCAGCTC > 1328 bp AAAAGTAACTAGTTTAGTTGCAACCTTTGTGAACAAGGACTTGCCTGCCTTTTTAGAGAA AGCTATGAAGAAGGAAATGTCTGCCATTGGACCTGCTGTGGTTCGCACAATAACACCAGC TATTGAGAAAACAATTTCTTCTGCCATCACTGATTCTTTCCAGAGAGGAGTAGGTGATAA GGCAGTAAATCAACTAGAGAAATCTGTTAGCTCAAAGCTTGAAGCTACTGTTGCTAGGCA CATCCAAGCGCAGTTTCAAACATCTGGCAAGCAAGCTCTGCAGGATGCATTAAAATCTAG TTTTGAAGCATCAGTGATTCCAGCCTTTGAAATGTCGTGCAAAACCATGTTTGAGCAAGT AGATTCTACCTTTCAGAAAGGACTGGTTGAACATTCAGCTGCAGCTCAACAACACTTTGA CTCTTCACATTCTCCATTGGCACATGCTTTGAGGGATTCTATAAATTCAGCTTCAACAAT TGCGCAGTCCTTGAGTGGAGAATTGGCTGAAGGCCAAAGAAAACTGATAGCCCTTGCGAC TGCAGGAGCAAATGCTAGTTCATTAAATCCTTTAGTTAGCCAACTAAGCAATGGGCCATT GGGTGCTCTTCATGAGAAGGTTGAGGTTCCTTTGGATCCTACAAAAGAACTGTCGAGATT GTTATCTGAAAGGAAATACGAGGAAGCTTTCACCGCTGCTTTACAGAGAAGTGATGTGAA CATTGTATCTTGGTTATGTTCTCAGGTCGATCTTAGAGCCGTTTTGGCAAATCCCCTTGC TTTGAGTCAAGGAGTACTGCTGTCCCTTTTGCAACAATTAGCATGTGATATCAATAAGGA TAGATCCCGGAAAATTGCATGGATGACTGAAGTGGCTGCTGCTGTAAATCCTGCAGACCC AATGATAGCGATGCACATACGGCCTATCTTTGAACAGGTGTATCAGATTTTGAACCATCA ACGGAGCTTGCCAACGGTTTCTCCAGTCGAGCTCACTGGCATTCGCATAATCATGCATCT TGTCAACTCCATGATGGTAACTTGTAAATGATTCTTCCCTCTTTTTCGTATGTAATTTTG

		TTCTGGCTTTTCAACACCCTAATGTGCAGATATAGAGCATGGAGCATAGATACACATCTC CTCTTTCATGTTTATTTCTGTAAAAGTCACCAAAACCGTGTAGATCCATTGTCGAATCTG TGAAATAGGCGTAGCATAGGCTCGCTCTCTTTTCTTTAAAAAAAAAAAAAAAGGGCGGCC GCGGGCCCATCGATTTTCCACCCGGGTGGGGTACCAGGTAAGTGTACCCAATTCGCCCTA TAGTGAGT
P4	not definied	CCNGGGATCTCATACAAACCATGGCACAAGCCGGATCTCTCTTGGTCGACACCGAGGC TCTTAGCCACATCTTCAATCTAATAGAAGCGTTTAGGGCATTTGATGCAGACAACGACGG GTTGATCTCGTCAGCTGAGGTTGGGGGGATCATGGGCTCGCTCGGGTACAACCTGAGTGA GGAAGACGTGAACATGATGATGGAGGAAGGTGACGCCGACAAAGACGGGCTGCTAAGCAT GGGGGAGTTCTTAGAGATGAATGCAAAGAATATGGATGTTGGGGAGTTGGGTAGCTACTT GAAGATTGCTCTTGAGGCTCTAAAGGCAGATGAGGATGATCTTGTGAGTGGGGAAGAGCT TTATGATGTGTTTGTGAATTTAGGGTTGGATGTTTCACTTGAGGATTCCATGGAAATCCA AGAATATGAACGAATTTCCGTTGCCGATGAAAATAGAACGCGAAAATGGATCCCGGGAAT TCCCGGGATCTGCCAAATACTTACACACTCCCAAGTCCTCATGGCCGCCTCCGCTGCCTC TGTCGGAGTCGTTAACCATGCACCGCTAAGCTTGAAAGGCTCTGGATCCACAACTTCGGT TCCAAGTTCAGTCTTCTTTGGGAACAGTTTG
P8-1	Hypothetical 61,5 kDa protein AL161576	AGGGAACAAAGCTGGAGCTCGCGCGCCTGCATGTCGACACTAGTGGATCCAAAGAATTCT TTGCAAGGCCACCACATGCAAGTAAAGTGTTAATCTTGTGCCCATGGGAATTGCAGTGCT CCACAATGTGGCGTGTACCATATGTTATAGCCTGTACGGTGGCAAGGTACAAAATAGAAA GCTGTTGCTCACTTGTGTCAAGTGTTAGCCCATAGATCACTCCTTTTGCTTTTGGATCCG AAATAGGAGACCTAATCCATGATGCAATCAAGTTCGTTGATAATCTCTAGCAGGGAAAGG AAGAAAAAGAACACCTGGAAAAATTACTCGGCAAAATGTCTCAGTCATTCATTCACCTAT TTCCATGAAAATCAGGAAGTATGTGTATATCTTCAGTCAAAGCAGCAAGAAATGGACTCT TCAGATCAACCACCAAATTCTCCAACAACTTGTTCAAGATATCAAACACAGAGATTTCTG AAAGATTAAAATTTAAAAAGAAAAGACAAAAAGAGAAGATGAACAATTCCAGATTAGACA CAATCATTTAACTTTTATGTATACCCTGCTTTAATCACAGTAGACGGCATCAGTATAATC AAATAACAAAAAGAGTCATACTTTGCGAAGCAGCACGATTTGCAAGATGAGGAGAGGCAA CATGATTTTGAATTATGTGATCAAGTAGAGCACCGGTAGCACTCTGACCACCTTCAGTAA GCCAATACTCAGGTACCATTGCTGACCAAAACGGTCCCCATACACCCGGAATAAAGAGCT TATCCCTTGAGACAGCCATATGGCAGGTAGATGTTCCACAAACCAGTGCCATACGATTCA ATATCATCTCCTTATTGAAACCTTCAGAATCAGAATCTTGCACGGGTACACTTTCCAACA CCCCAACTCCACCTGCATGAGCATCAATCAGTGAAACACCAACAGGGATGCCAGCAACCA TACCCAGTTCCTTCGCAGCTACTGGAGTCAGACCAGAACCCAAGGGGTGACCAGGGAATG CTACACTTCTATTTTCGCATGATGACCATCGATAAGATCTCTTAATCCAATTTCCTCCCA GAAATCATCATCCCATCCACAAGCCTCCATATTTCGGGAATCCTTTTCATTGTACTGTGA CATGTGTGCATGACCAAGATAGGTCCATTTGCACACGGTGGTGCATAGACTGCGTGTGTC ATCACCAGTGGCCCTAGATCCCGGGAATTCAAAAAGCTTCTCGAGAGTACTTCTAGAGCG GCCGCGGGCCCATCGATTTTCCACCCGGGTGGGGTACCAGGTAAGTGTACCCATTCGCCC ATAGTGAGT
P16-1	Hypothetical 35,4 kDa protein AL078470	AGGGAACAAAAGCTGGAGCTCGCGCGCCTGCATGTCGACACTAGTGGATCCAAAGAATTC CCGGGATAAACCTTTCCCCACCCTATCCCTTCCTTTCCTCTCTTTCCGCCTCTGCACAAT GTCAACTTCTGGCGACATTGTCATCTCTTCAACCACCCGCATCGGCTGGATCGGCACCGG CATTATGGGCTGCTTAATGTGCCCTCACATCATCAAAGCTGGCTACAAACTCACCGTCTT CAATCGCACCATCTCAAAAGCCCAACCCCTCCTTGATTTAGGCGCCAATTTAGCCTTGTC CCCTCTTGATGTTGCTGCTCAGTCCGATGTCGTTTTCTCCGTCGTCGGTTACCCCTCCGA CGTCCGCTCTGTCCTCCTCGACCCCAATTCCGGCGCCCTTGCTGGCCTTCGCCCTGGCGG TGTCCTTATCGACATGACCACTTCCGAAGCATCACTCGCCTCTGAAGTCGCTGCCGCTGC AACTGCCAAGGGTTGTTGGGCAATCGATGCTCCGGTGTCAGGCGGCAATCGTGGAGCCAA GAACGCCACTCTGGCGATCTTTGCTGGTGGCGATGAAGGCGTAGTCCGGCGATTGAGCCC GTTGTTTTCTCTTTTAGGGAAAGTGAATTACATGGGGGAAAGTGGGAAAGGGCAGTTTAC AAAGCTAGCGAATCAGATCATCATAGCTTCATCATTGGTGGGACTAGTGGAAGGGATGAT TTATGCTAAGAAAGCAGGGCTTGATGTGGGTTTGTTCTTGAATGCTATATCAACAGGAGG AGCTGGATCAAAATCTTTGGATTTATATGGAAGTAAGATTTTGGAAAGGGATTTTCAACC TGGGGGTTTTGTGGAACATTTTGTGAAGGATATTGGAATTTGTTTGAAGGACTGTCAGAA TATGGGGATTGCTCTTCCTGGATTGACGTTAGCACACCAGCTTTATGTTTCGCTTAAGGC TTATGGGGATGGGAATTTAGGTGTTCATGCACTGATTTTGTCTCTGGAACGGCTTAATAA TGTTTCACTTGATGACGGCGATAGCGGGGGCAGTGGTTGTTGATTATGCCTCCATTACAA AGACCACTAATCCTTGATTCTAAGCTATTTGGTTATACCAATAAAGAAAGAGGTCATCCA

		TATAAGTTTCAACTTTCCAATTTGTGGTTGTTTACTACAATTATAAATAAGGAACTTATG AAGTTATAAAAAAAAAAAA

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clone name	Homology	Nucleotide sequence
29-1	14-3-3	GCCAAGCTCGAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCGCGCGCCTGCAGG TCGACACTAGTGGATCCAAAGAATTCGGCACGAGGAAAAACTCAATCTCTCTCTCTCT CACACACTTTCTCTCTCCAATGGCGGCAACACTAGGCAGAGACCAGTACGTCTACATGGC CAAGCTCGCCGAGCAAGCCGAGCGCTACGAGGAGATGGTCAACTTCATGGAGAAGCTCGT CACAGGCGCAACACCATCCTCCGAACTCACCGTCGAAGAACGTAACCTCCTCTCCGTGGC TTACAAGAACGTGATCGGATCCCTGCGCGCCGCGTGGCGCATCGTGTCCTCGATCGAGCA GAAGGAAGAGAGCAGGAAGAACGAGGAACACGTGGCGCTCGTGAAGGACTACAGATCTAA AGTGGAGACGGAGCTCTCCTCCGTCTGCGAAGGGATACTAAAGCTTCTCGAGGAGAATCT GATTCCCTCTGCGGCGGCGAGCGAGTCGAAGGTGTTTTATCTGAAGATGAAGGGGGATTA CCATCGGTACATGGCGGAGTTTAAGGCCGGTGAGGAGAGGAAAGCGGCGGCGGAGGATAC TATGGTTGCTTACAAGGCTGCTCAGGATATTGCAGCTGCTGATATGGCGCCTACTCATCC GATTAGGCTTGGTTTGGCGTTGAATTTCTCGGTGTTTTACTATGAGATTCTTAATTCTTC GGACAAAGCTTGTGACATGGCCAACAGGCTTTTTGAAGAAGCCATAGCTGAGCTTGA CACTCTTGGAGAAGAGTCGTACAAAGACAGCACTCTCATTATGCAGCTGCTCAGGGACAA TCTTACCCTCTGGACCTCTGATATGCAGGAGCAGATGGACGAGGCATGAGGAGCTTGAGG AAGGGAGAGTGTTATGAGCCGTGTTTCCGCCACCAATTTCGATCTCAACATCCCCCGTAA CCTTTGCTCAAAACTGTTGAACCAAAAAAAAA
48-1	Thioredoxin h 1	GGCACGAGGCAAACAGAGAGAGAGAACAATGGCCGCAACAGCAGAATTGATCCCAGCAGG AGAAGTGATCGCTTGCCACACCGTCGAGGACTGGAACAACAAGCTCAAGGCAGCCAAAGA ATCCAACAAACTGATTGTGATAGACTTTACAGCAGTGTGGTGCCCACCTTGCCGTTTCAT TGCACCCATCTTTGTCGAGCTCGCTAAGAAGCACCTTGATGTTGTCTTCTTCAAGGTCGA CGTCGATGAATTGGCCACTGTTGCAAAGGAGTTCGATGTTCAGGCCATGCCGACCTTTGT CTACATGAAAGGGGAAGAGAAGCTCGACAAGGTTGTTGGTGCTGCGAAGGAGGAAATCGA AGCCAAGCTTTTGAAGCACTCTCAAGTTGCTGCCGCTTGATTATCCTTCTTCTGCTTCTA TTTGCTAATGTGCT
54-1	RIO1 family protein	TTAACCAAAACAAAAGATGGAAGAAGTTATAGC AGAGCCGCCTCCGACTATCTCTCCGGCGATTGTGGATTACGACGAGCAGATGGAAGACGA TGACGACGACTCCGATCTTTCTATCTCGTCAGACTCCGACATAGGTGAAGCTCTAGACTG GCTAGACGGCAAAGACGACGACGAGCTCATCGGTGGCGGCTTCTCTCTGCACGCTCGTCG TCCCAACGCCCACGGCGGAAATGGAGCTAGGCCTAACTCGGGCGCTCTTCAGCCTCTTTC TAACAAGGCTCAGAAGCTTTCCAGCCATGTCCGCGCGTCCCCGTTGGAGGCGTGGGAAGG TAGAGTTAAAGTCGGGATGTCGAACTCCGTGACGACTGCGATACGTGGGAGTCTCAGAGA CACGGAGATCGGGAGGAGCAGGAACACTGATAAAGCAGACAGGGCGACGGTTGAACAGGC GCTTGATCCGAGGACACGTATGGTGTTGTTTAGGATGCTTAATCGTGGTGTGTTTAATGA TGTTAATGGATGTATTTCCACAGGCAAAGAAGCTAATGTTTATCATGCTACAAAGTTTGA TGGTGCGGAGCTTGCAATAAAAGTGTACAAGACATCAGTTCTGGTCTTCAAGGATCGAGA TCGATATGTACAAGGAGATTACCGTTTCAGACATGGCTACTGTAAGCATAATCCCCGGAA GATGGTGAAGACGTGGGCTGAGAAAGAACACAGAAATCTTAAGAGGCTTCAGGCAGCAGG AATTAGATGTCCAGTTCCTATACTTCTACGACTTCATGTTCTAGTCATGGAGTTTATAGG AAGAGATGGCTGGGCTGCACCGCGTCTCAAGGATGCTGCACTATCACTAGACAAGTTACG CGAGTCTTATCTAGAGTTGATAATCCAAATGAGAACATTATATCAGAAGTGCAAACTGGT GCATGGAGATCTCAGTGAGTATAACATACTTTATTTCGAGGGGCACCTTTATATAATTGA TGTTTCACAATCTGTGGATCTGGACCATCCTCTTGCCCTCACTTTTCTACGGGAAGATTG TGACCACGTTTCAGATTTTTTCAAGAAACATGGTGTTGCTGTAATGACCATAAGGGAGCT ATTTGACTTCATAGTGGACCCC
40-1	plastocyanin	GGCACGAGGGCAAAACATAATAAAATGGCATCAGTTACATC AGCTACCGTTGCATTCCCATGTTTTACCGGCCTCAAGCTCGCCGTCAACGCCAAACCCAC GGCGGTATCCACCGCCGCCATCAAATCTCCCTCCAAAGCGGCGCCTAAGCTATCCGTGAA GTCTTCTCTTAAAGATTTTGGCGTCATGGCCGTTGCAGCAGCAGCTTCCATCGCTTTGGC

		CGGGAATGCGATGGCCATTGAGATACTCTTAGGATCTGATGATGGTGGTCTTGTTTTCGT ACCAAGCGACTTCACGGTGGCTAAAGGAGAGAAGATCGTGTTCAAGAACAACGCAGGGTA CCCACACAACGTGGTGTTCGACGAAGACGAGATCCCTAGTGGCGTGGACGCGAGCAAGAT CTCGATGGACGAGCAAGCGCTTCTCAATGGTGCGGGAGAGACGTACGAGGTTACGTTGAC AGAACCTGGCTCTTACAGTTTCTACTGTGTGCCCCACCAGGGAGCTGGTATGGTTGGAAA GCTCACCGTCAACTAAACTGTGAG
47-1	Double stranded DNA-binding protein	TAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCGCGCGCCTGCAGGTCGACACTAGTGG ATCCAAAGAATTCGGCAAGAGGGAAGACTGCTCCAATGGCTGATCCTGAACTAGAAGCTA TTCGACAGAGGAGAATGCAAGAGCTCATGGCTCATTATGGCGCTGGGAAGCAGGGGAGTC AGCAGAATCCAGATCAAGAGAGAGCACAGGAAGATGCTAAAAGGGAAGCTGATGAACGTA GACNAATGATGCTTAGTCAAATCTTGTCTTCCCAAGCCCGTGAGAGAATTGCTCGAATTG CCCTGGTGAAACCTGAGAAAGCTAGAGGTGTAGAGGATGTTATTTTGAGGGCTGCTCAAA TGGGACAGATTGTTGAGAAGGTTTCTGAGGAGCGACTTATAACGCTGCTGGAACAAATAA ACAGCCAGACAACCAAACAGACTAAAGTCACGATCCAGAGGCGTCGTGGGGTGGATGACG ATTAGGAAGAAGGAGACTATGCTATGCTTTTGTATATCTATGGAGATCTCACTGTTGAGT TGGTGAGTTCACTAAGATGTACTTTAAGTGTTACTGTGAAATATGTTTATTATGAGGGAA TCTTGATGATCTTCCAAGTGATTGGGATGAGATCATAAAAGTAATCTGAAACTGAAACTC TGCATTTTATCTTTTTGGCATTTCCTCTGTGAACCATCTCTATCTTCCTCTCCTGTCCTC GCATTG
12-1	Unknown protein gi|30725324|gb|AAP37684.1|	GAAACGTCCATGTTAGCTAACAAGCTCAAGAACATTCATGACCA TCTCGCTGAGACCTTGAGGCTTTGCTACCGTCACATAGAGAAGCAGAGGAGCTCAGAATC CTTAAAGATGTTGCATTCTCTATTCGACACAACACACATTGATAACATGAAGATTCTCAC GGCCCTCATTCATCCTAAAAACCACACCATGCCATTGCAAGATGGTTTAACCAAGAGAAA GGTTCACTTGGACGTGTTGAGGAGGAAGACAGTGTTGTTGCTAATATCTGACCTCAACAT ATTGCAAGACGAGCTATCGATATTCGAGCAAATCTACACAGAATCAAGGAGCAGCTTGTT GGGAGCCGATGGTAAGAGTCATATGCCGTACGAGGTTGTGTGGGTTCCGATAGTGGACCC TATCGAAGATTACGAAAGATCACCGAGTCTGCAGAAGAAATTCGAGGCTCTCCGTGCACC TATGCCATGGTACACAGTGGATAGTCCAAAGCTGGTAGAGAGACATGTGGTGGAGTTTAT GAGAGAGAGATGGCATTTCATGAACAAGCCAATACTTGTGGTGCTTGACCCACAAGGCAA CGAAGCTAGTCTCAATGCGCTTCACATGATCTGGATTTGGGGGACTGAAGCTTTCCCTTT CACTAGAGCTCGTGA
26-1	Unknown protein 20148268|gb|AY081463.1|	TCGGCACGAGGTATACATATATTAAGAAAAATGCTT GACACACTTATCGGTGGGCACGGGCACGGGTTCGGCCACAAGGATCACATAAGCAATGGT TACGGCTTCGAGGGGCACAATGAGTTTGTGTCTCATGAGGACTCAGAAAGGAGCCACTTC GATCGTCAATCCCGCTACGACCACCAAATGAGGCTACCTGCTAACCACGGCCGTCCACCC ATGGCTCGCATGCCTCCCTGTGATGAAGAAGAGTCAGACGATGATGAGTTCATTAAGAGA AGCCGTAGCCACCACACTACTGTGTTGCCGCATCACCAACAGAAACCCCATATGAACTTC ATGCCCCCACCTCCGTTGTCTCAGCCTCACCACAACGGAAAGATGGGTAATGGATGGCAG GGAAGGCATGAAGATGGATACCATGGCGGGCACGGGATGCAGCAGCACGGTGGTCACGGC ATGCAGCAGCATGGTGGGCATGGGATGCCACAACACGGTGGGCACGGGATGCAGCAGCAT GGTGGGCACGGAATGCAGCACCACGATGGGCACGGGATGCAGCACCACGATGGGCACGGT GGGCACGGTATGCAGCAGCACGGGGCGCACGGGATGCAGCACCACGATGTGCACGGTATG CAGCAGCACGGGGCGCACGGGATGAAGCACAGGACGGCTTATGGGTCCTCA
43-2	not defined	GGCACGAGGAATGCTTGACACACTTATCGGTGGGCACGGGCACGGGTTCGGCCACAAGGA TCACATAAGCAATGGTTACGGCTTCGAGGGGCACAATGAGTTTGTGTCTCATGAGGACTC AGAAAGGAGCCACTTCGATCGTCAATCCCGCTACGACCACCAAATGAGGCTACCTGCTAA CCACGGCCGTCCACCCATGGCTCGCATGCCTCCCTGTGATGAAGAAGAGTCAGACGATGA TGAGTTCATTAAGAGAAGCCGTAGCCACCACACTACTGTGTTGCCGCATCACCAACAGAA ACCCCATATGAACTTCATGCCCCCACCTCCGTTGTCTCAGCCTCACCACAACGGAAAGAT GGGTAATGGATGGCAGGGAAGGCATGAAGATGGATACCATGGCGGGCACGGGATGCAGCA GCACGGTGGTCACGGCATGCAGCAGCATGGTGGGCATGGGATGCCACAACACGGTGGGCA CGGGATGCAGCAGCATGGTGGGCACGGAATGCAGCACCACGATGGGCACGGGATGCAGCA CCACGATGGGCACGGTGGGCACGGTATGCAGCAGCACGGGGCGCACGGGATGCAGCACCA CGATGTGCACGGTATGCAGCAGCACGGGGCGCACGGGATGAAGCACCAGGACAGGCTTAT GGGTCCTCAGATTCACCACATCATGTCTACAT TACCATGGCGGGCACGGGATGCAGCAGCACGGTGGTCACGGCATGCAGCAGCATGGTGGG CATGGGATGCCACAACACGGTGGGCACGGGATGCAGCAGCATGGTGGGCACGGAATGCAG

		CACCACGATGGGCACGGGATGCAGCACCACGATGGGCACGGTGGGCACGGTATGCAGCAG CACGGGGCGCACGGGATGCAGCACCACGATGTGCACGGTATGCAGCAGCACGGGGCGCAC GGGATGAAGCACCAGGACAGGCTTATGGGTCCTCAGATTCCACCACATCATGTCTACATG AACCCCAACCATGGAAGTGGTAGTGGCCGTACAGTAATGGTGAAGGCTTCGGAGAACTGG CGAATGAGCAAGAGCACCGGCGGCCACCACAAGGTCGGGTGGGGGAGTAAGGGACTCTAA GTGCACCACAATACTCTGCCGGTTCAAATAAGAAAGTAATGATTATATTTCCGAAATAAT AATTTTATAAGATGTATGTGTGTGATGTTTCAGTAACGTACCAAAACCAAACGCTTAGAC TCTTCATAAAAAATGTTTTCGTTTCATGAACTTATGATTGTTAGGGGGTGTTGTTGTTGT TGCTTGACTTAAATAAAATGTTTTCGTTTCATGACCAAAAAAAAAAAAAAAAAAAAACT

C

Table V Nucleotide sequences isolated from expression libraries screening. **A**- sequences from C. sativus library, **B**- sequences from C. maxima library, **C**- sequences from B. napus library.

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