Identification and characterization of small RNAs from the phloem of *Brassica napus*

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Summary

Systemic signalling is indispensable for the coordination of diverse physiological processes during development, defence and nutrient allocation. Indirect evidence suggests that plant small RNAs (smRNAs) could be involved in long-distance information transfer via the vasculature of the plant. Analyses of the smRNA complements of vascular exudates from oilseed rape (*Brassica napus*) showed that xylem sap is devoid of RNA, whereas phloem sap contained a large number of smRNAs. In addition to 32 annotated microRNAs (miRNAs) from 18 different families that could be identified and approved, a set of unknown smRNAs, predominantly of 21 and 24 nucleotides in length, was obtained, and selected candidates were found to be highly abundant in phloem sap. Moreover, we could demonstrate that the levels of three miRNAs known to respond to nutrient deprivation in non-vascular tissue, miR395 (sulphate), miR398 (copper) and miR399 (phosphate), were increased in phloem sap during the growth of plants under the respective starvation conditions. Interestingly, only mature miRNA molecules were found to be stress responsive, demonstrating that single-stranded sense miRNAs are most likely to represent the physiologically relevant molecules. The strong responses in the phloem suggest a role of miRNAs in systemic information transfer via this long-distance transport system.

Keywords: *Brassica napus*, microRNA, phloem sap, copper starvation, phosphate starvation, sulphur starvation.

Introduction

Higher plants are multicellular organisms that are dependent on a highly organized and complex regulation of gene expression to allow coordinated growth and development. Recently, different classes of small RNAs (smRNAs) were found to influence many levels of gene regulation. Small regulatory RNAs are molecules of ~19–25 nucleotides (nt) in length (Bartel, 2004) that control mRNA stability, translation or target epigenetic modifications to specific regions of the genome (Finnegan and Matzke, 2003), by complementary binding to target nucleic acids. Based on differences in their biogenesis and action, smRNAs have been grouped into different classes, the most prominent being short interfering (si) and micro (mi) RNAs. Although siRNAs act in defence of the genome, miRNAs mainly guide growth and development by regulating endogenous target genes. However, some miRNAs have also been shown to be involved in the coordination of nutrient homeostasis (Aung *et al.*, 2006; Bari *et al.*, 2006; Jones-Rhoades and Bartel, 2004; Sunkar *et al.*, 2006; Yamasaki *et al.*, 2007).

Biogenesis of siRNA and miRNA are complicated processes that require multiple enzyme-catalyzed steps to form mature molecules (Sunkar and Zhu, 2007). siRNAs are processed by DICER-like enzymes (DCL2, DCL3 and DCL4) from long and perfectly complementary double-stranded (ds) RNA molecules that can originate from viruses, aberrant RNAs or endogenous antisense gene pairs. These ds molecules are themselves produced by RNA-dependent RNA polymerase (RDR) activities. The resultant 21- and 24-nt siRNAs are incorporated into a ribonucleoprotein complex, known as the RNA-induced silencing complex (RISC), where they guide the cleavage of target mRNAs (Sunkar and Zhu, 2007).

In contrast, miRNAs are derived from longer single-stranded hairpin precursor molecules that are processed to duplexes of mature miRNAs and miRNA*s without perfect sequence complementarity, by the enzyme DCL1, presumably in the nucleus (Jones-Rhoades *et al.*, 2006). After export to the cytosol miRNA*s are degraded, whereas mature

miRNAs enter RISC to regulate the expression of target genes (Jones-Rhoades *et al.*, 2006), which generally show near-perfect sequence complementarity to mature miRNAs (Rhoades *et al.*, 2002).

Recent studies suggest that smRNAs can act as potential information-transmitting molecules that can move between cells, and can even spread systemically via the long-distance transport systems (Chen and Kim, 2006; Jorgensen, 2002; Kehr and Buhtz, 2007; Lough and Lucas, 2006; Yoo *et al.*, 2004). Consistently, it could be demonstrated that plant siRNAs do not only act at the site of synthesis, but are additionally mobile between cells (Himber *et al.*, 2003). There are indications that siRNAs can move 10–15 cells without amplification, probably as molecules of the 21-nt class, whereas movement over greater distances requires an amplification of the original signal (Himber *et al.*, 2003). Also, phloem mobility of siRNAs has been suggested since the first discovery of the systemic spread of post-transcriptional gene silencing (Hamilton and Baulcombe, 1999; Lucas *et al.*, 2001; Palauqui *et al.*, 1997; Tournier *et al.*, 2006), and 24–25-nt siRNA molecules are thought to be sufficient for spreading the long-distance signal (Hamilton *et al.*, 2002). However, confirmation that siRNAs really constitute the phloem-translocated signal propagated during gene silencing is still elusive (MacDiarmid, 2005).

It is currently unknown if similar or different short- and long-distance translocation mechanisms for miRNAs or other smRNAs exist, or if their mode of action is completely cell-autonomous (Voinnet, 2005). That a recent study identified siRNAs, but also four known miRNAs, in the phloem of cucurbits argues for long-distance mobility. The same study provides indications that the phloem miRNA population is mainly single stranded, and that the transport is probably mediated by specific RNA-binding proteins, because pure diffusion through plasmodesmata seems to be insufficient to move smRNA species in microinjection experiments (Yoo *et al.*, 2004). Moreover, the accumulation of different miRNAs in vascular tissue led to the suggestion that miRNAs could be mobile signals (Aung *et al.*, 2006; Bari *et al.*, 2006; Juarez *et al.*, 2004; Valoczi *et al.*, 2006). However, direct evidence for the transport and the functions of miRNAs in the phloem are as yet missing.

The present study had three major aims: (i) to perform a comprehensive identification of the smRNA complement of the long-distance transport fluids from *Brassica napus*; (ii) to verify a physiological relevance of phloem smRNAs by analysing the levels of specific, nutrient stress-induced miRNAs under altered environmental conditions; (iii) to examine in which form miRNAs occur in the phloem.

Our results reveal that a specific set of smRNAs is present in phloem samples from *B. napus*. We obtained sequences from a large number of as yet unknown smRNAs, mainly of 21- and 24-nt in size, and some candidates could be confirmed to occur exclusively in the phloem as sense and reverse complementary strands. We could additionally identify 32 miRNAs belonging to 18 different annotated families from *A. thaliana*. Surprisingly, we could detect not only the mature miRNAs but also the miRNA*s. Strand-specific RNase digests indicated that both molecules exist in the phloem as single strands.

Nutrient starvation experiments demonstrate that the levels of miR395, miR398 and miR399 in the phloem are strongly increased in response to sulphate, copper or phosphate starvation, respectively. The failure to detect an increase in the level of miRNA*s corresponding to the high rise in mature miRNA clearly indicates that single-stranded sense miRNAs are the physiologically relevant miRNA species within the phloem.

Results and discussion

Brassica napus *phloem, but not xylem sap, contains a characteristic RNA pattern*

One major aim of the present study was to establish the RNA compositions of xylem and phloem samples from *B. napus*. This species has the advantage of being closely related to the model plant *Arabidopsis thaliana*, and additionally allows obtaining phloem and xylem samples in comparably large quantities and of relatively high purity (Giavalisco *et al.*, 2006; Kehr *et al.*, 2005).

It has been shown that xylem sap collected by root pressure exudation from stems is not cross-contaminated with phloem sap (Kehr *et al.*, 2005). Such xylem sap samples were free of RNA, even when large millilitre quantities were analysed. In contrast, phloem sap contained a characteristic RNA pattern at a total concentration of about 0.03 $\mu g\ \mu l^{-1}$. Bioanalyzer measurements of this RNA showed a specific size distribution, with two large peaks probably resembling ribosomal RNA (rRNA; Figure 1a). As it is well accepted that mature, transporting sieve elements contain either no or only a very low number of non-functional ribosomes, we analysed phloem samples for the presence of two abundant photosynthesis-related transcripts (*rubisco* and *chlorophyll a/b binding protein*) that are not expected to occur in sieve elements, and could ensure that our phloem samples are not significantly contaminated with the content of broken photosynthetic cells (Figure 1d). This supports an earlier study (Giavalisco *et al.*, 2006) that also demonstrated that the technique for *Brassica* phloem sampling results in samples largely enriched in phloem sap. Surprisingly, significant proportions of ribosomal transcripts were also detected within phloem exudates of different plant species, e.g. ricinus seedlings (Doering-Saad *et al.*, 2006) or pumpkin (Ruiz-Medrano *et al.*, 2007), indicating that ribosomal RNAs are indeed an authentic phloem sap component. We

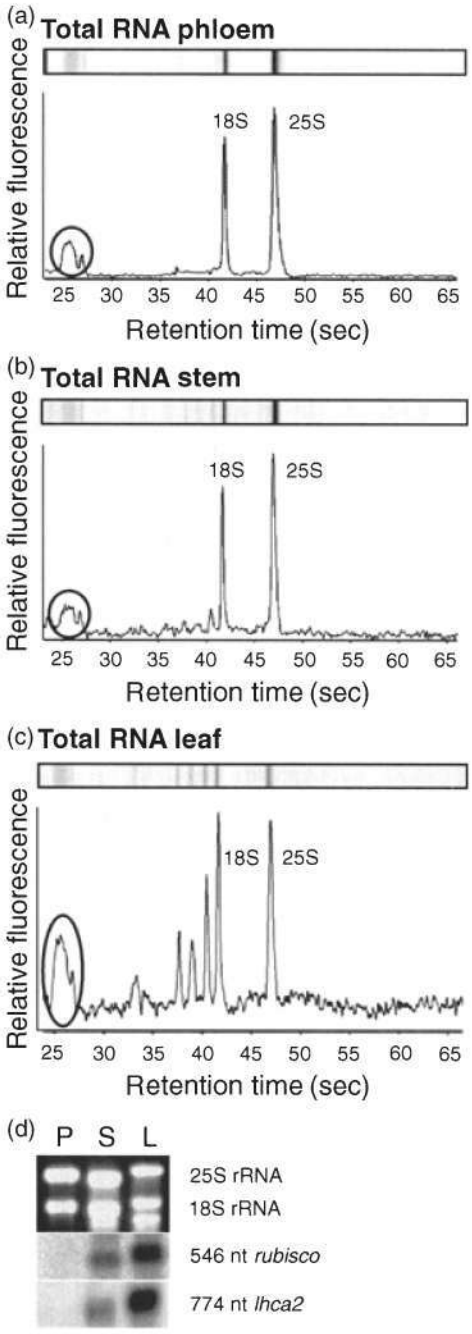

Figure 1. Characterization of the RNA composition of *Brassica napus* phloem sap.

RNA size distribution of (a) phloem sap (55 ng), (b) inflorescence stems (19 ng) and (c) leaves (22 ng) determined with an Agilent Bioanalyzer 2100 equipped with an RNA Nano chip. Low-molecular-weight RNA fractions are encircled. 18S and 25S: ribosomal peaks. The corresponding gel-like picture of separated total RNA is displayed above the electropherogram.

(d) RNA gel blot analysis of two photosynthesis-related mRNAs in leaf (L), inflorescence stem (S) and phloem sap (P) RNA samples. Transcripts of the small subunit of ribulose bisphosphate carboxylase/oxygenase (*rubisco*) and the chlorophyll *a*/*b* binding protein (*lhca2*) were both strongly expressed in leaves and inflorescence stems, whereas they were not detectable in phloem samples (the blot was re-probed with *lhca2*).

assume that the observed rRNA is likely to represent a remnant from still differentiating sieve elements in growing regions of the plant.

Total RNA from inflorescence stem tissue, obtained at the same location as the phloem samples, and of leaves displayed similar RNA profiles, but with additional small peaks, presumably caused by plastidic rRNAs (Figure 1b,c). In addition, phloem sap contained a reasonable fraction of low-molecular-weight RNAs (Figure 1), as did all other tissues analysed, normally including, dependent on the tissue type, 5.8S, 5S, transfer RNA (tRNA), miRNAs, siRNAs and other smRNAs (Masotti and Preckel, 2006).

Phloem samples from Brassica napus *contain smRNAs*

To obtain a comprehensive picture of the small RNA complement in the phloem, the low-molecular-weight RNA of phloem sap was enriched, manually cloned and sequenced (Chappell *et al.*, 2005), or, in two out of three independent samples derived from different plant sets, were subjected to high-throughput pyrosequencing. These combined approaches generated five phloem sap smRNA data sets containing >30 000 non-redundant sequences ranging in size from 20 to 25 nt, with 24 nt representing the most common length (Table 1; Figure S1). This is in accordance with earlier phloem results and comparable pyrosequencing experiments from other plant tissues, where 23–24-nt RNAs were also the most abundant species (Henderson *et al.*, 2006; Lu *et al.*, 2006; Yoo *et al.*, 2004).

To identify sequences that represent authentic and reliable constituents of phloem sap, only the ones occurring in at least two independent data sets and showing a length of 20–25 nt were initially considered. Using these criteria, a number of 859 smRNAs could be extracted from the raw data sets (Table S3). To obtain information about the similarity of the resultant smRNAs, BLAST searches using NCBI and the miRNA Registry database (miRBase) were performed.

An overview of the results is presented in Table 2. A portion of the sequences could be identified as different known plant miRNAs (see below). Most of the others

Table 1 Number of redundant and non-redundant sequences obtained from the individual phloem sap small RNA (smRNA) sequencing approaches

Phloem libraries of gel-fractionated smRNAs	Strategy	Total no of sequences (20–25 nt)	Non-redundant sequences (20–25 nt)
1	Pyrosequencing	21 024	18 662
2		15 508	13 366
1	Cloning	242	61
2		260	57
3		301	294
Total of all libraries		37 335	32 262

Table 2 Classification of small RNA (smRNA) sequences that occurred in at least two different data sets

Groups of smRNA species	No. sequences (20–25 nt)	Percentage related to 859 filtered sequences
Ribosomal RNA	64	7
Known plant micro RNA	214	25
Homology to other database entries	350	41
No homology	231	27

showed high similarity to mainly *Brassica rapa* clones (listed in Table S3), whereas only 7% were identified as rRNAs, which are generally seen as the background in cloning and deep-sequencing approaches (Reinhart *et al.*, 2002; Ruby *et al.*, 2006).

To confirm the phloem presence of some candidates, we analysed their abundance in phloem, leaf and stem samples by RNA gel blots. We selected four candidates: two abundant sequences of 24 nt in length (BnsmRNA_1 that showed no match to database entries and BnsmRNA_2 that was identical to the *B. rapa* clone AC189207; cloned 10 and 109 times, respectively), and two other sequences (BnsmRNA_3 and BnsmRNA_4; cloned 18 and six times, respectively) displaying features of potential miRNAs, as they were 21-nt long and started with a uridine at the 5′-end (Tang, 2005). All four candidates could be exclusively detected in phloem samples, but in no other tissue analysed (Figure 2), indicating that these smRNAs are authentic.

Additionally, we designed probes against the perfect reverse antisense sequences to test whether any of the four candidates belongs to the class of siRNAs that show, in contrast to miRNA*s, a complete complementarity of both strands. We could detect reverse strands for both selected 24-nt smRNAs (BnsmRNA_1 and BnsmRNA_2) and for one of the 21-nt (BnsmRNA_4) smRNAs, all exclusively in the phloem (Figure 2). This indicates that these three small RNAs might represent as yet unknown siRNAs. The antisense sequence of BnsmRNA_3 was not detectable, indicating that this candidate is no siRNA but could represent novel miRNA.

A large number of known miRNAs are contained in the phloem

Database searches identified 32 annotated plant miRNAs belonging to 18 different families. The miRNA homologues occurred between 1 and almost 60 times, as displayed in Figure 3. Seventeen sequences appeared in at least two different phloem samples (Table 3). The observation that not all miRNAs were detected in all samples could result from the incomplete coverage of

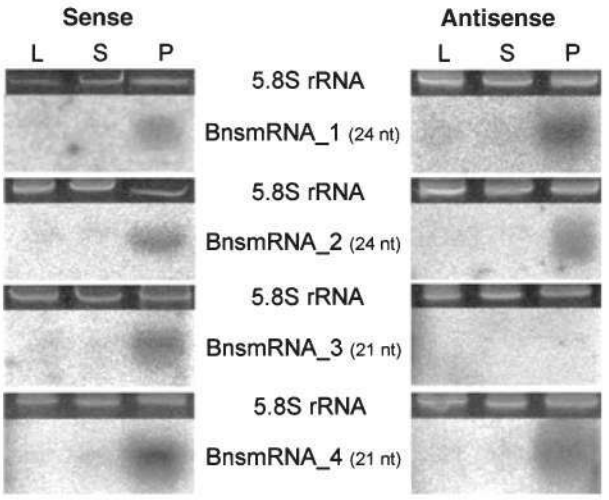

Figure 2. RNA gel blot detection of four selected unknown small RNAs (smRNAs) (BnsmRNA_1, BnsmRNA_2, BnsmRNA_3 and BnsmRNA_4) and the reverse complementary antisense sequences in the phloem compared with leaf (L) and inflorescence stem (S) RNA samples. RNA gel blot analysis confirmed the presence of sequences of BnsmRNA_1, BnsmRNA_2 and BnsmRNA_4 exclusively in the phloem (P), as no signals in the other tissues could be detected. For BnsmRNA_3, no reverse complementary sequence could be detected.

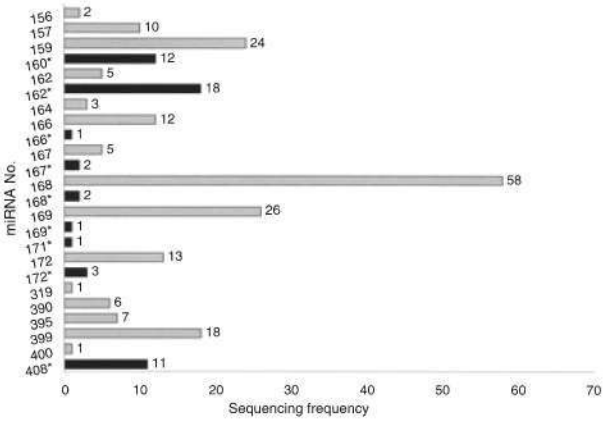

Figure 3. Abundance of mature micro RNA (miRNA) sequences (grey bars) and miRNA*s (black bars) in phloem sap of *Brassica napus*, summarised from all five data sets.

scarce RNAs by the applied sequencing procedure (abundant miRNAs that were frequently sequenced usually also appeared in more than one sample, Table 3), and additionally from biological variation between the three different plant sets used for obtaining samples. Therefore, we cannot exclude that additional miRNAs that have not yet been found in this study occur in the phloem (see for example miRNA 398 that was not included in the sequencing data sets but was detectable by RNA gel blots, discussed below).

Table 3 Summary of sequences from the phloem of *Brassica napus* that correspond to known *Arabidopsis thaliana* micro RNAs (miRNAs)

Sequence	miRNA	Organism	Size (nt)	No. of sequences	Presence in × data sets	RNA gel blot		Predicted target gene [reference]
						Mature strand	*Strand	
*T*TGACAGAAGAGAGTGAGCAC	miR156a-f	*A.th.*	21	1	1	✔	n.d.	Squamosa promoter-binding protein[1]
TTGACAGAAGATAGAGAGCA	miR156a,b,c	*A.th.*	20	1	1			
TTGACAGAAGATAGAGAGCAC	miR157a,b,c	*A.th.*	21	10	2		✔	
TTTGGATTGAAGGGAGCTCTA	miR159a	*A.th.*	21	24	2	✔	✔	GAMYB transcription factors[1]
GCGTATGAGGAGCCATGCATA	miR160a*	*A.th.*	21	12	2	✔	✔	Auxin response factors (ARF)[1]
TCGATAAACCTCTGCATCCAG	miR162a,b	*A.th.*	21	5	2	✔	✔	DICER-LIKE 1 (DCL1)[1]
GGAGGCAGCGGTTCATCGATC	miR162a*,b*	*A.th.*	21	18	2			
TGGAGAAGCAGGGCACGTGCA	miR164a,b	*A.th.*	21	2	2	✔	✔	NAC domain transcription factors[1]
TGGAGAAGCAGGGCACGTGCG	miR164c	*A.th.*	21	1	1			
TCGGACCAGGCTTCATTCCCC	miR166a-g	*A.th.*	21	12	2	✔	✔	HD-ZIP transcription factors[1]
GAATGTTGTCTGGCTCGAGG	miR166b*	*G.max*	20	1	1			
TGAAGCTGCCAGCATGATCTA	miR167a,b	*A.th.*	21	5	1	✔	✔	Auxin response factors (ARF)[1]
GATCATGTTCGCAGTTTCACC	miR167a*	*A.th.*	21	2	1			
TCGCTTGGTGCAGGTCGGGAA	miR168a,b	*A.th.*	21	58	4	✔	✔	ARGONAUTE 1[1]
CCCGCCTTGCATCAACTGAAT	miR168a*	*A.th.*	21	2	1			
CAGCCAAGGATGACTTGCCGA	miR169a	*A.th.*	21	9	3	✔	✔	HAP2-like transcripton factors[1]
CAGCCAAGGATGACTTGCCGG	miR169b,c	*A.th.*	21	3	2			
TAGCCAAGGATGACTTGCCTG	miR169h-n	*A.th.*	21	2	2			
CAGCCAAGGATGACTTGCCG	miR169a,b,c	*A.th.*	20	12	2			
CAAGTTGACTTTGGCTCTGT	miR169e*	*A.th.*	20	1	1			
AGATATTAGTGCGGTTCAATC	miR171b*	*A.th.*	21	1	1	✔	n.d.	Scarecrow-like transcription factors[1]
AGAATCTTGATGATGCTGCAG	miR172c,d	*A.th.*	21	3	1	✔	✔	APETALA 2 transcription factors[2]
AGAATCTTGATGATGCTGCAT	miR172a,b	*A.th.*	21	7	2			
GGAATCTTGATGATGCTGCAT	miR172e	*A.th.*	21	3	1			
GCAGCACCATTAAGAT	miR172b*	*A.th.*	16	3	1			
TTGGACTGAAGGGAGCTCCC*C*	miR319a,b	*A.th.*	21	1	1	✔	✔	TCP transcription factors[3]
AAGCTCAGGAGGGATAGCGCC	miR390a,b	*A.th.*	21	6	2	✔	✔	TAS3[4]
CTGAAGTGTTTGGGGGAACTC	miR395a,d,e	*A.th.*	21	2	1	✔	✔	ATP sulfurylase APS4[5]
CTGAAGTGTTTGGGGGGACTC	miR395b,c,f	*A.th.*	21	5	2			
TGCCAAAGGAGAGTTGCCCTG	miR399b,c	*A.th.*	21	18	2	✔	✔	UBQ conjugating enzyme[6]
TATGAGAGTATTATAAGTCAC	miR400	*A.th.*	21	1	1	✔	n.d.	Pentatricopeptide repeat-containing protein[7]
CAGGGAACAAGCAGAGCATGG	miR408*	*A.th.*	21	11	3	✔	✔	Peptide chain release factor, laccase[7]

A tick indicates a successful confirmation of the miRNA candidate by RNA gel blotting. Italic letters in the sequence column mark the two nucleotides that were not identical to the annotated database sequences.
A.th., Arabidopsis thaliana; *G. max, Glycine max*; n.d., not detectable.
[1]Reinhart *et al.* (2002), [2]Park *et al.* (2002), [3]Palatnik *et al.* (2003), [4]Allen *et al.* (2005), [5]Jones-Rhoades and Bartel (2004), [6]Aung *et al.* (2006), [7]Sunkar and Zhu (2004).

Our data show that phloem sap contains more than the four miRNAs found previously in the phloem of pumpkin (Yoo *et al.*, 2004), three of which were also present in our *Brassica* databases (miR156, miR159and miR167). From the fourth miRNA, only the corresponding miR171* strand could be identified.

Most of the *Brassica* miRNA sequences corresponded to mature Arabidopsis miRNA homologues, but, in nine instances, the sequences corresponded to the complementary (miRNA*) side of the stem loop structure of the miRNA precursors.

The presence of all identified miRNAs could be confirmed by RNA gel blot analyses (Figure 4a), only miR156/157 and miR319/159 could not be distinguished. Interestingly, the patterns of miRNAs were clearly different when comparing phloem with inflorescence stem or leaf tissues, in that miRNAs were over- (miR156/157, miR168, miR169 and miR395) or under-represented (miR167, miR171, miR172 and miR400) in the phloem (Figure 4a). A second distinctive feature of the phloem miRNAs was electrophoretic mobility: the stem or leaf-derived miRNAs were predominantly 21 nt in size, whereas some (e.g. miR156, miR164 and miR390) migrated consistently with a mixture of ~24- and 21-nt species, the larger species being more abundant in the phloem. This indicates that either miRNA molecules of different sizes occur or that the 21-nt species is modified, e.g. by uridylation (Li *et al.*, 2005).

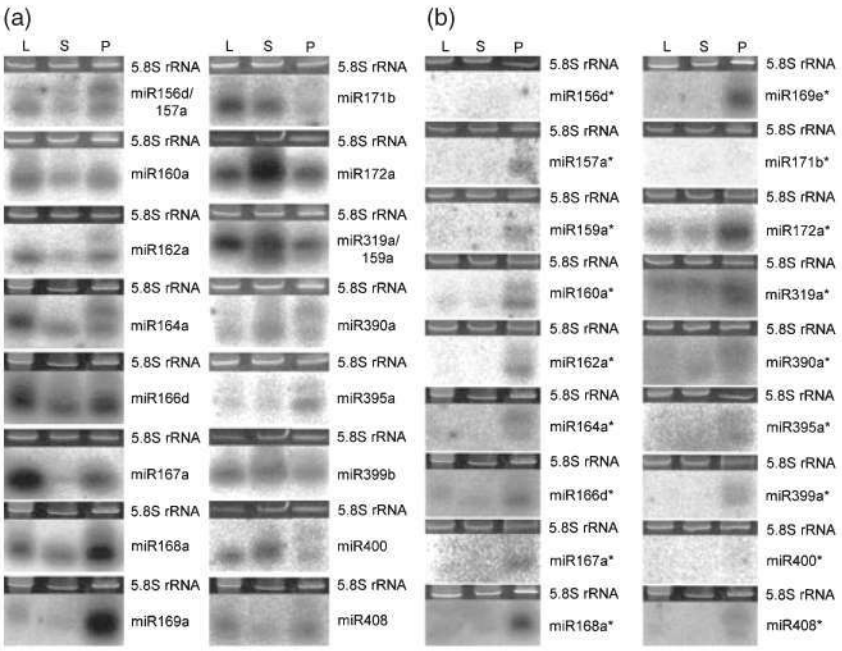

Figure 4. Analysis of the identified micro RNAs (miRNAs) in samples from phloem (P), leaves (L) and inflorescence stems (S) from *Brassica napus* by RNA gel blotting.
The results for mature miRNAs (a) and their corresponding miRNA*s (b) are presented. Note that the mature miRNAs miR156/157 and miR319/159 could not be distinguished by RNA gel blotting because of nearly absolute sequence identity. Loading control: 5.8S ribosomal RNA (rRNA). Approximately 15–20 μg of total RNA from each tissue was loaded on 15% polyacrylamide gels.

*Phloem miRNAs are present as mature miRNA and miRNA*s*

As we obtained sequences not only from mature miRNAs but also from some miRNA*s, we tested for the presence of all miRNA*s corresponding to the identified mature phloem miRNAs (Figure 4b). In contrast to double-stranded siRNAs, miRNAs and miRNA*s originate from a single precursor molecule. It is known that mature miRNAs are highly conserved between different, even unrelated, plant species (Zhang *et al.*, 2006), and a recent study confirmed a high similarity of *B. napus* miRNAs to those of other plants (Xie *et al.*, 2007). In contrast, miRNA*s show no perfect reverse complementarity with mature miRNA molecules (Reinhart *et al.*, 2002), and can substantially vary between species (Bonnet *et al.*, 2004), which must be taken into account in probe design. We therefore designed DNA oligonucleotides against the complementary parts of the Arabidopsis precursor molecules.

Figure 4b shows that most miRNA*s (except for miR156, miR171 and miR400) were detectable with the Arabidopsis probes, indicating that they are not that different in both species. Interestingly, the miRNA*s were over-represented relative to the leaf and shoot samples with 15/18 different probes (Figure 4b).

The failure to detect miRNA*s in the other tissues analysed is in accordance with earlier studies that found miRNA*s in tissues of wild-type Arabidopsis plants to be nearly undetectable by RNA gel blot analyses (Dunoyer *et al.*, 2004; Kurihara and Watanabe, 2004). In contrast to our results on *Brassica*, Yoo *et al.* (2004) could not detect the phloem presence of four miRNA*s in samples from pumpkin. This is probably caused by low miRNA abundance and detection sensitivity, as these four miRNA*s also yielded no (miR156 and miR171) or only low (miR159 and miR167) signals in our experiments (Figure 4b).

The same study suggested that miRNAs in cucurbits occur as single-stranded molecules within the phloem. However, because our sequencing approaches and RNA gel blot analyses indicated that phloem sap contains mature miRNAs as well as miRNA*s, we analysed if miRNA/miRNA* duplexes are present in the phloem transport stream of *Brassica*. To clarify this, we performed single-strand- or double-strand-specific RNase digests on isolated total phloem sap RNA (Appendix S1) indicating that single strands are predominant (Figure S2). From the RNA digests alone, however, it could not be excluded that formerly double-stranded RNA molecules were irreversibly separated during the RNA isolation procedure. Nevertheless, our nutrient stress experiments (see below), showing that only the mature miRNAs and not the miRNA*s increase during stress treatments, also strongly suggest that mature miRNA single strands, and not miRNA/miRNA* duplexes, are the physiological relevant molecular species.

We were not able to detect any precursor molecules using oligoprobes against sequences of the stem loop structure by RNA gel blots in any of the tissues we analysed. Because of the rapid turnover of the precursor molecules, which brings their levels below the detection limit of the blotting method, precursors are normally not detectable in plant tissues of wild-type plants (Reinhart *et al.*, 2002). However, the occurrence of primary miRNA

transcripts or other miRNA precursor molecules in the phloem seems rather unlikely, as there is as yet no evidence for the presence of miRNA processing enzymes in sieve elements.

Phloem miRNAs are not only present, but respond to changes in growth conditions

The 18 different miRNA families found in the phloem of *Brassica* can target genes of different functional categories (Table 3). Most (11) play diverse roles in plant development by decreasing the expression of several transcription factors. It is estimated that about 80% of the predicted miRNA targets are transcription factors regulating developmental fate (Bartel and Bartel, 2003). The other miRNAs are involved in the regulation of smRNA biosynthesis pathways or nutrient stress responses (Table 3).

An accumulation of different miRNAs in vascular tissue of several plant species previously led to the suggestion that miRNAs might be mobile signals (Aung *et al.*, 2006; Bari *et al.*, 2006; Juarez *et al.*, 2004; Valoczi *et al.*, 2006).

Therefore, we decided to follow the levels of three miRNAs that are known to respond to nutrient availability, miR395, miR398 and miR399, in nutrient stress experiments. If these miRNAs do indeed function as long-distance information carriers, one would consequently expect their accumulation in phloem samples after the inducing trigger. However, a response of the levels of any miRNA within phloem sap has not yet been directly demonstrated.

miR395 was shown to be strongly induced by low sulphate concentrations in the growth medium in non-vascular tissues of Arabidopsis (Jones-Rhoades and Bartel, 2004). The detailed function of miR395 in sulphur metabolism is still unclear, but two potential targets, the ATP sulfurylase APS4 (Jones-Rhoades and Bartel, 2004) and the sulphate transporter *AtSULTR2;1* (Allen *et al.*, 2005; Chiou, 2007) were experimentally confirmed. To check whether miR395 abundance in the phloem of *Brassica* is also affected by sulphur supply, we grew plants in hydroponic cultures with medium omitting sulphate for 2 weeks and tested for the level of miR395. We could observe a significant increase of miR395 in the phloem of plants grown under sulphur depletion, and the observed increase was much stronger in the phloem compared with root, stem or leaf tissue (Figure 5a).

Although we could not find miR398 in our data sets, we chose copper starvation as a second stress treatment, because miR398 was recently shown to respond to copper deprivation in non-vascular tissues of Arabidopsis plants (Sunkar *et al.*, 2006; Yamasaki *et al.*, 2007). Consistent with the results from miR395, we could observe an upregulation of miR398 not only in leaf, stem and root

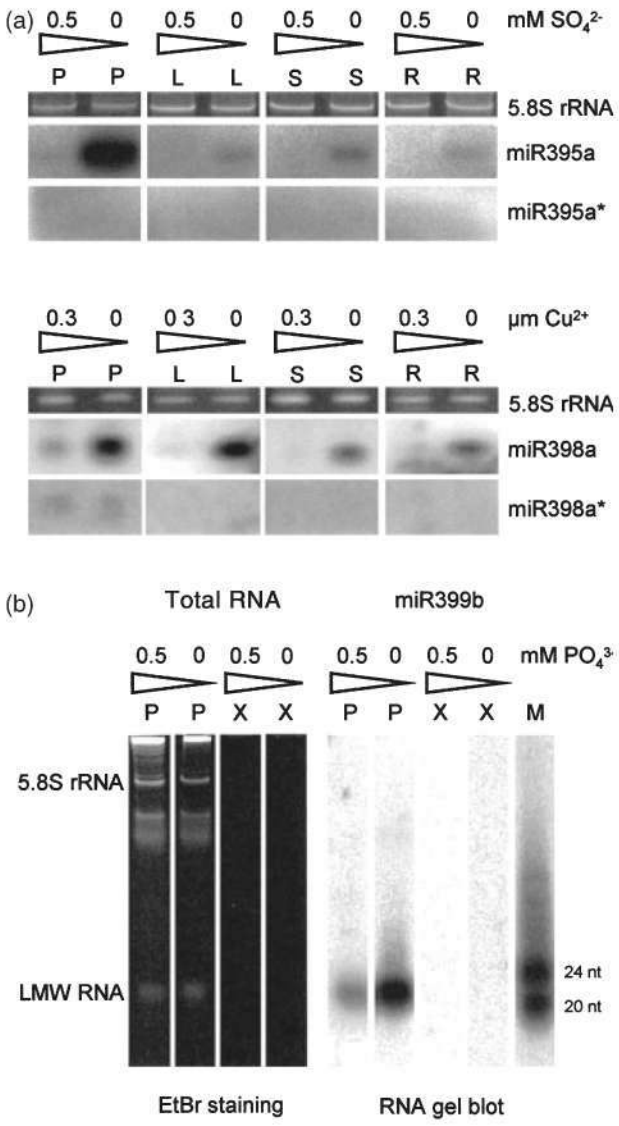

Figure 5. Analysis of the stress-responsive microRNAs (miR395, miR398 and miR399).

(a) Response in the levels of mature miR395 and miR398, and their corresponding miRNA*s, to low sulphate and copper conditions, respectively, in phloem sap (P), leaf (L), flowering stem (S) and root (R) tissue. RNA gel blot detection showed a drastic increase in the phloem, whereas the other tissues showed only a slight induction of mature miR395. miR398 shows a strong signal in all copper-starved tissues, including the phloem, whereas the miR398* was only detectable in the phloem, and no induction of the miRNA* strand could be observed. miR395* could not be detected in sulphur stressed tissue or controls of any tissue analysed (but was detectable in the phloem of soil-grown plants, demonstrating the functionality of the probe, see Figure 5b), indicating that the mature miR395 and miR398 are the functionally relevant miRNA species in the phloem.

(b) Detection of miR399 in phloem (P) and xylem (X) sap of phosphate-deficient plants. The left panel shows ethidium bromide (EtBr) stained total RNA of phloem (P) and xylem (X) samples separated on 15% denaturing polyacrylamide gels. Phloem sap showed a characteristic RNA pattern, whereas xylem sap samples were devoid of RNA. RNA gel blot analysis (right panel) detected miR399 exclusively in phloem, but not in xylem samples. M: RNA oligonucleotides of 20 and 24 nt in size (Ambion) used as molecular size standard; LMW RNA: low-molecular-weight RNA fraction.

tissue, but also in phloem sap (Figure 5a) under copper deprivation.

Also, the mature form of miR399 increased drastically in low-phosphate media (Figure 5b), which is in agreement with earlier studies from other tissues in Arabidopsis that showed an upregulation of miR399 in low-phosphate conditions (Bari *et al.*, 2006; Chiou *et al.*, 2006; Fujii *et al.*, 2005). This led us to a more detailed examination of the role that miR399 plays during the response to low-phosphate levels (Pant *et al.*, 2007).

Next, we investigated whether mature miRNAs, miRNA*s or both can be considered as the transmitting information molecules in nutrient stress. Therefore, we re-analysed the blots from the starvation experiments using miRNA*-specific probes. In hydroponically grown plants, the miRNA*s were hardly detectable and there was no increase corresponding to the rise of mature miRNA observed (Figure 5a). This indicates that single-stranded mature miRNAs are the molecular species involved in transmitting information about the nutrient status of the plant.

Finally, although we found no RNA in xylem sap of plants grown with full nutrition, we additionally investigated a possible increase of miR399 under phosphate starvation. Compared with the strong signal observed in phloem samples, miR399 was absent in xylem sap of both unstressed and stressed plants (Figure 5b), indicating that the xylem is not involved in miRNA signalling.

Conclusions

Our high-throughput sequencing approach demonstrated that a broad range of small RNAs, including known miRNAs among a majority of sequences with as yet unknown relevance, are abundant components of phloem sap in *B. napus*.

Although it would be tempting to assume that all of the detected smRNAs are involved in long-distance communication, it seems more realistic that some of them represent mRNA breakdown products, remnants from developing sieve elements or normal outflow of miRNAs accumulating in the adjacent companion cells.

However, the present study provides convincing evidence that specific phloem smRNAs can indeed play important roles in the coordination of nutrient allocation between plant organs, and that single-stranded mature miRNAs are the molecules with functional importance. miRNAs could even be long-distance 'nutrient deficiency signals' that regulate the nutrient status of the plant via the phloem. Clearly, further investigations will be required to identify the origin of phloem smRNAs, to establish their import and transport mechanisms, and, most importantly, to elucidate their exact physiological functions.

Experimental procedures

Plant growth on soil

Brassica napus plants (cv. Drakkar; Serasem GIE, la Chapelle d'Armentiers, France; http://www.invivo-group.com/02_metiers/01semenc/serasem.htm) were grown in 19-cm diameter pots containing sterilized soil (Einheitserde® Typ T) in a greenhouse under controlled conditions (16-h light, 8-h dark, 25°C day, 20°C night, 55% relative air humidity). Plants were automatically watered three times a day with tap water containing Hakaphos® spezial as fertilizer.

Nutrient stress experiments

For hydroponic growth, seeds of *B. napus* (cv. Drakkar) wild-type plants were germinated on wet Whatman paper for 1 week. Germ buds were transferred to plastic boxes containing nutrient medium for 10 weeks. Nutrient medium: 0.6 mmol l^{-1} NH_4NO_3, 1 mmol l^{-1} $Ca(NO_3)_2{\cdot}4H_2O$, 0.04 mmol l^{-1} FeEDTA, 0.5 mmol l^{-1} K_2HPO_4, 0.5 mmol l^{-1} K_2SO_4, 0.4 mmol l^{-1} $Mg(NO_3)_2{\cdot}6H_2O$. Micro nutrients added: 0.8 μmol l^{-1} $ZnSO_4{\cdot}7H_2O$, 9 μmol l^{-1} $MnCl_2{\cdot}4H_2O$, 0.1 μmol l^{-1} $Na_2MoO_4{\cdot}2H_2O$, 23 μmol l^{-1} H_3BO_4, 0.3 μmol l^{-1} $CuSO_4$. The pH level was adjusted to 4.7 with 37% HCl. Nutrient solutions were changed after 4 weeks, and were then renewed once a week. After 5–6 weeks, media were constantly oxygenated by an aquarium air pump (sera GmbH, http://www.sera.de). Sulphur, copper and phosphate starvation were applied for 2 weeks before flowering started by changing to a medium without sulphur, copper or phosphate, respectively. Here, 0.5 mmol l^{-1} K_2SO_4 was substituted by 0.5 mmol l^{-1} K_2HPO_4, and instead of $ZnSO_4{\cdot}7H_2O$ and $CuSO_4$ as micro nutrients, 1 μmol l^{-1} $ZnCl_2$ and 1 μmol l^{-1} $CuCl_2$ were added for low sulphate experiments. For copper deprivation, the 0.3 μmol l^{-1} $CuSO_4$ was omitted from the full nutrient solution. For low phosphate experiments, 0.5 mmol l^{-1} K_2HPO_4 was replaced by 0.5 mmol l^{-1} K_2SO_4.

Phloem and xylem sap sampling

Phloem sampling from *B. napus* plants was performed as described earlier (Giavalisco *et al.*, 2006). Phloem sap was collected from between four and eight small punctures into the inflorescence stems. After discarding the first droplets to avoid contamination, samples of between 500 μl and 1.5 ml of phloem sap from three independent sets of plants were obtained for the different sequencing experiments, yielding about 10–50 μg of total RNA. About 500 μl of phloem sap was used for the detections.

Xylem sap from rape plants was sampled by root pressure exudation from stems cut near the soil level, as described previously (Buhtz *et al.*, 2004; Kehr and Rep, 2006; Kehr *et al.*, 2005). A 2-ml volume of root pressure exudate from plants grown on hydroponic cultures was collected directly into Trizol LS reagent (Invitrogen, http://www.invitrogen.com).

RNA isolation

Total RNA from phloem and xylem sap was isolated by Trizol LS reagent (Invitrogen) according to the manufacturer's instructions. RNA from 100 mg of frozen material of leaf and stem tissue of *B. napus* was extracted using Trizol. Total RNA from all samples was then resolved in 25 μl of diethylpyrocarbonate

(DEPC)-treated water. RNA concentrations were measured photometrically with a Biophotometer (Eppendorf, http://www.eppendorf.com). RNA quality was controlled by formaldehyde gel electrophoresis, or by microfluidics-based lab-on-a-chip technology (Bioanalyzer 2100; Agilent Technologies, http://www.agilent.com), which allows RNA quality and quantity to be determined by electrophoretic size fractionation of fluorescently labelled RNA molecules in a gel matrix detected by laser-induced fluorescence. RNA samples were prepared and measured according to the manufacturer's instructions using the Agilent RNA 6000 Nano Kit and the RNA 6000 Nano marker for size estimation and quantification.

Sequencing of smRNA species

Small RNA cloning was performed exactly by the method described by Chappell *et al.* (2005) following the basic protocol, excepting the concatamerization step that was omitted. Briefly, total RNA samples were separated on 15% denaturing polyacrylamide gels, and gel parts containing smRNAs (20- and 30-nt long) were excised and eluted. After recovery from the gel, purified smRNA was precipitated, redissolved and sequentially ligated to 5′- and 3′-adapters (Table S1). Subsequently, reverse transcription and PCR amplification were performed using specific primer pairs (Table S1).

For manual cloning, aliquots of each sample were digested with *Eco*RI and *Nco*I restriction enzymes, subsequently ligated in a pGEM-T-easy vector (Roche Rapid DNA Ligation Kit; Roche, http://www.roche.com) and transformed into TOP10 competent *Escherichia coli* cells (Invitrogen). Colonies were screened for cDNA inserts and a *SAP/Exo*I digest was performed to clean the samples for the automated sequencing (John Innes Centre Genome Laboratory, http://jicgenomelab.co.uk). The samples were prepared for sequencing following the instructions of the BigDye terminator cycle sequencing kit (Applied Biosystems, http://www.appliedbiosystems.com). For the high-throughput sequencing approach, an aliquot of the PCR amplified cDNA library was sequenced by 454 Life Sciences (http://www.454.com).

Database searches and identification

All data sets were first processed (subtraction of adapter sequences and reduction to 20–25-nt long sequences) and then used for BLAST sequence homology searches on the miRNA Registry website (release 8.2) at http://microrna.sanger.ac.uk/sequences/index.shtml, for the identification of miRNAs. For the analysis of unknown sequences reliably occurring in more than one experiment, data sets were compared using Microsoft Office Access 2003 (Microsoft, http://www.microsoft.com), and the resultant RNA sequences were used to perform nucleotide–nucleotide BLAST searches for short, nearly exact matches (NCBI, http://www.ncbi.nlm.nih.gov/BLAST).

RNA gel blot analysis

Gel blot analyses were performed on 15% denaturing urea gels according to the method described by Chappell *et al.* (2005) using the DNA oligonucleotides (MWG-Biotech, http://www.mwg-biotech.com) listed in Table S2. In the case of miRNA*s, probes were designed against the annotated sequences of the Arabidopsis pre-miRNA molecules.

For end labelling, 2 μl of 10 μM oligonucleotides were mixed with 2 μl of 10 × reaction buffer B (supplied with polynucleotide kinase; Fermentas, http://www.fermentas.com) and 10 μl of water before adding 5 μl of radioactive γ-[^{32}P]ATP and 10 U (1 μl) of polynucleotide kinase enzyme (Fermentas). The probes were purified from non-incorporated γ-[^{32}P]ATP nucleotides by using MicroSpin™ G-25 columns (GE Healthcare, http://www.gehealthcare.com) following the manufacturer's instructions. The blots were hybridized overnight at 40°C and washed two times with washing solution [2 × SSC, 0.1% (w/v) SDS] at 40°C.

For the detection of *rubisco* and *lhca2* transcripts, total RNA from inflorescence stems and leaves was Trizol-isolated and RNA samples were additionally purified using the RNeasy Plant Mini Kit with on-column DNAse 1 digest (Qiagen, http://www.qiagen.com). RNA was then separated on formaldehyde-containing 1.5% LE (low electroendosmosis) agarose (Biozym, http://www.biozym.com) gels, which were blotted and UV cross-linked. Radioactively labelled probes were prepared by using the Rediprime II Random Prime Labelling system (GE Healthcare) following the manufacturer's instructions. For this purpose, cDNA synthesis with total RNA from flowering stem (~ 400 ng) was performed in a 20-μl volume of 1× M-MLV RT reaction buffer (Promega, http://www.promega.com), containing 2.5 μM oligo(dT)$_{20}$-nucleotides (Invitrogen), 0.5 mM dNTP-Mix (Promega), 5 mM DTT (Invitrogen), 40 U RNaseOut RNase Inhibitor (Invitrogen) and 200 U of M-MLV Reverse Transcriptase (Promega) under the following conditions: 45 min at 50°C, 15 min at 70°C, 5 min at 37°C. A 2-μl volume of the reverse transcriptase reaction was used for PCR amplification with gene-specific primer pairs (Table S2). PCR amplification was carried out in 50 μl of 1 × GoTaq® DNA Polymerase reaction buffer (Promega) containing 1 mM $MgCl_2$ (Invitrogen), 0.2 mM dNTP-Mix (Promega), 0.2 μM of each primer and 2 U of GoTaq® DNA Polymerase (Promega) with following thermocycler conditions: 30 s at 94°C, 30 s at 55°C, 1 min at 72°C (35 cycles) and a 10-min end-elongation step at 72°C. Amplified PCR products were separated on 2% agarose gels, and the fragments with expected sizes (*rubisco*, 546 nt; *lhca2*, 774 nt) were eluted with the Qiaquick PCR Purification Kit (Qiagen) following the manufacturer's protocol. A 5-μl volume of the isolated gene-specific fragment and radioactive α-[^{32}P]dCTP were used for labelling. The probes were purified from non-incorporated α-[^{32}P]CTP nucleotides by using MicroSpin™ G-50 columns (GE Healthcare). The RNA gel blots were hybridized overnight at 63°C and washed two times with washing solution [2 × SSC, 0.1% (w/v) SDS] at 63°C. Hybridization signals for all RNA gel blots were detected by phosphoimaging overnight, and were visualized by using a BAS Reader-1500 Imaging Analyzer and TINA 2.10 software (Fujifilm, http://www.fujifilm.com). All RNA gel blots were repeated at least twice and showed consistent results.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Size distribution of the smRNA data sets from *Brassica napus* phloem sap obtained by cloning and high-throughput pyrosequencing.

Figure S2. Single- and double-strand-specific RNase digests with total phloem sap RNA.

Table S1. 5′ and 3′ adapters and primer sequences used for small RNA cloning and pyrosequencing.

Table S2. Oligodeoxynucleotides used for RNA gel blot detection.

Table S3. Summary of all short sequences in the size range of 20–25 nucleotides that were found in at least two independent phloem small RNA data sets.

Appendix S1. Experimental procedures.

This material is available as part of the online article from http://www.blackwell-synergy.com

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