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Phloem transport of amino acids in two *Brassica napus* L. genotypes and one *B. carinata* genotype in relation to their seed protein content

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Abstract. In order to investigate the relationship between the amino acid concentration in the phloem sap of leaves and the protein content in seeds, two *Brassica napus* genotypes and one *B. carinata* genotype with low, medium and high seed protein contents were analyzed. Phloem sap was collected from the *B. napus* winter rapeseed breeding line DSV15 with 19% protein of dry weight in the seeds, the spring cultivar 'Duplo' with 25% protein in the seeds and from the *B. carinata* line BRA1151/90 with 39% protein in the seeds by using the aphid-stylet technique. The total amino acid contents measured in the phloem varied considerably among the three genotypes analysed, and correlated positively with their respective seed protein contents. The total amino acid-to-sucrose ratio was lowest in *B. napus* line DSV15 which had the lowest seed protein content and highest in the *B. carinata* line BRA1151/90 which had the highest seed protein content. The amino-N translocation in the phloem during the light period was about 2-fold higher in the *B. carinata* line BRA1151/90 than in the *B. napus* lines Dulpo and DSV15. Predominant amino acids in the phloem were glutamine and glutamate, followed by serine, aspartate, and threonine. The amino acid patterns in the leaves resembled those in the phloem, although their absolute concentrations were higher in the phloem than in the cytosol of mesophyll tissue. Furthermore, the concentration gradient of amino acids between the cytosol of mesophyll cells and the phloem was higher in the *B. carinata* line BRA1151/90 than in the *B. napus* lines Duplo and DSV15. These results lead to the conclusion that the phloem translocation of amino-N and the phloem loading process of amino acids are decisive factors for the protein content in the seeds of *Brassica* species.

Key words: Amino acid – *Brassica* – Phloem transport – Seed (protein content)

Abbreviation: NR = nitrate reductase

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Introduction

The seeds of oilseed rape (*Brassica napus*) are characterised by an oil content of 40–50% and a protein content of 20–30% (Finlayson 1976; Uppström 1995). Related *Brassica* species like *B. juncea* and *B. carinata* usually have a lower oil and a higher protein content in their seeds. Early investigations of genetically segregating plant material revealed a negative correlation between the oil and protein contents in the seed (Grami et al. 1977). The mechanism and regulation of the accumulation of storage proteins and storage lipids in the seeds of *Brassica* species is still poorly understood. The increase in the seed oil content at the expense of the seed protein content is one major goal in breeding programs. Understanding the biochemical mechanisms involved in the partitioning of oil and protein synthesis in the growing embryo could be useful for modifying the ratio between oil and protein contents in the seeds of oilseed rape. Furthermore, genotypes with a low seed protein content are expected to utilize nitrogen (N) fertilizer more efficiently to obtain a high seed yield, resulting in an improved N-use efficiency (Sattelmacher et al. 1994).

During seed filling a considerable part of the nitrogen is provided by the vegetative parts of the plant, such as leaves and stem. The amount of nitrogen remobilization from senescing tissue is a central issue for the nutrient budget in seed crops (Feller and Keist 1986). Nitrogen is translocated mainly via the phloem in the form of amino acids. The total amino acid concentrations in the phloem sap of different crops, such as rice, barley, maize or sugar beet, range between 50 and 200 mM (Fukumurita and Chino 1982; Lohaus et al. 1995, 1998; Winzer et al. 1996). A comparison of different hybrids of sugar and fodder beet revealed a correlation between the concentrations of amino acids in the phloem sap and the corresponding concentrations in the taproots (Winzer et al. 1996).

Several studies already have analyzed the extent and mechanism of oil and protein storage in rape seeds (Clercq et al. 1990; Kohno-Murase et al. 1994; Zou et al. 1997), however, any measurements of phloem

transport were lacking. The following study was performed with two genotypes of *B. napus* (DSV15, Duplo) and one of *B. carinata* (BRA1151/90). These genotypes were selected because of the difference in their seed oil and protein contents: DSV15 has a low, Duplo a medium and BRA1151/90 a high protein content. Phloem sap was collected from these genotypes and analyzed for sucrose and amino acid concentrations as well as for amino-N translocation rates. The results were compared with the seed protein content. For determination of the amino acid concentration gradients between the cytosol of mesophyll cells and the phloem the corresponding concentrations in the cytosolic compartment of mesophyll tissue were analyzed by subcellular analysis of leaves using non-aqueous fractionation (Gerhardt et al. 1987).

Materials and methods

Plant material

Seeds from *Brassica napus* L. genotypes were taken partly from the departmental collection in Göttingen, and partly provided by the breeding company 'Deutsche Sortenvereinigung' (Lippstadt, Germany). Seeds of *B. carinata* accessions were provided by the Institute for Plant Genetics and Crop Plant Research (Genbank, Gatersleben, Germany). *Brassica napus*, *B. carinata*, and *Pisum sativum* L. were grown in compost soil in a greenhouse with supplementary light. The light-dark period was 15 h light with a photon flux density of 300–900 μmol m^{-2} s^{-1} and 9 h darkness. Experiments were performed with plants at the late flowering stage.

Determination of the content and amino acid composition of seed protein

The analysis of oil and protein contents in *B. napus* (DSV15, Duplo), *B. carinata* (BRA1151/90) and *P. sativum* (cv. Kleine Rheinländerin) was analyzed by near infrared reflectance spectroscopy. Hydrolysation, derivatisation and HPLC analysis of amino acids of the seed proteins were done as described in Möllers et al. (1996).

Collection of phloem sap

Phloem sap was obtained from severed stylets of the oat-bird cherry aphid, *Rhopalosiphum padi* L. About 10 aphids were caged for 2–3 h on the mid-portion of a leaf and their stylets then severed by a laser beam (Lohaus et al. 1995). The exudating phloem sap was collected in micro-capillaries. The front edge of the micro-capillary was in close contact with the leaf surface and the end was surrounded by a cap to prevent evaporation of the phloem sap. The humidity around the capillary was adjusted to about 80%. Under these conditions reference capillaries did not show any evaporation. Exudation rates per stylet were about 100 nl h^{-1} (as determined by drawing the exudate sieve tube sap into a calibrated 0.5-μl disposable micro-capillary). Samples were taken in the second half of the light period. The samples were diluted with HPLC water to a volume of 100 μl and stored at −80 °C.

Leaf and seed extraction

To determine the tissue contents of sucrose and free amino acids, leaf and seed material was sampled. After shock-freezing in liquid nitrogen, approx. 0.25 g of tissue was ground in a mortar in liquid nitrogen. A mixture of 5 ml chloroform:methanol (3:7, v/v) was added to the powder and the sample was homogenized until complete thawing and kept on ice for 30 min. The homogenate was then extracted twice with 3 ml water. The aqueous phases were combined and evaporated in a rotatory evaporator. The dried residue was dissolved in 2 ml ultrapure H_2O (Millipore) and stored at −80 °C. In earlier experiments with sugar beet extracts, the recovery during the extraction procedure of sucrose, glutamate, glutamine, aspartate, asparagine and serine was between 85 and 112% (Winzer et al. 1996).

Determination of vacuolar and cytosolic metabolite levels by non-aqueous fractionation

Leaf samples were ground in a mortar to a fine powder while kept in liquid nitrogen and lyophilized. The leaf homogenate was suspended in heptane-tetrachloroethylene (C_2Cl_4) (density = 1.28 g ml^{-1}). Two milliliters of this suspension was added to a centrifugation tube containing, from bottom to top, 2 ml heptane-C_2Cl_4 (density = 1.60 g ml^{-1}) and 15 ml of heptane-C_2Cl_4 with an exponential density gradient between 1.51 and 1.28 g ml^{-1}. After centrifugation (25 000 *g*, 2.5 h) the separated material was collected in six or seven fractions, of which aliquots were taken for the determination of the marker enzymes NADP-glyceraldehyde phosphate dehydrogenase, phosphoenolpyruvate-carboxylase and α-mannosidase as markers for chloroplasts, cytosol and vacuoles, respectively, and of metabolites. The chloroplastic material was found to be concentrated in the middle region of the gradient, the cytosolic material appeared in the lower region whereas the vacuolar material was mainly found in the fraction of highest density. For determination of metabolite concentrations in the gradient fractions, chloroform methanol extracts were prepared (see above).

For the evaluation of the subcellular distribution of sucrose and amino acids between the stromal, cytosolic, and vacuolar compartments, a calculation procedure according to Riens et al. (1991) was used. This calculation method is based on the assumption that the metabolites are confined to the three compartments designated by the corresponding marker enzymes. The evaluation is done by a computer program testing all possible cases for the distribution of a certain metabolite between the three compartments at intervals of 1%. The program calculated which possibility yields the best fit (agreement) with the experimental results. To avoid the results being falsified by analytical errors, the calculations are usually based on mean values obtained from measurements of at least three density gradient fractionations.

Analysis of sugar and amino acids

Amino acids were analysed by HPLC (Pharmacia/LKB) using the fluorescent *o*-phthaldialdehyde pre-column derivatisation method according to Riens et al. (1991). Sugar concentrations were determined by HPLC. The anion-exchange column (CarboPac PA10; Dionex, Idstein, Germany) was eluted isocratically with 100 mM sodium hydroxide buffer (1 ml min^{-1}). Sugars were detected by a pulse amperometric detector with a gold electrode (ESA, Model 5200; Coulochem II, Bedford, Mass., USA).

Measurements of CO_2

Net photosynthesis of leaves in the light period and respiration in the dark period were measured with a portable infrared gas analyzer (LCA3; ADC, Hoddesdon, UK).

Extraction of soluble proteins and determination of nitrate reductase activity

Frozen leaf tissue was ground rapidly in a pre-chilled mortar with extraction buffer containing 100 mM Hepes-KOH (pH 7.5), 10%

glycerine (v/v), 1 mM EDTA, 0.1% Triton X-100 (v/v), 0.5% BSA (w/v), 5 mM Mg-(acetate)$_2$, 5 μM Na_2MoO_4, 1% polyvinylpolypyrrsolidone (w/v), 5 mM DTT, 25 μM Leupeptin, 20 μM FAD, 0.5 mM phenylmethylsulfonylfluoride at a ratio of 0.2 g leaves per ml buffer. Nitrate reductase (NR) activity was determined as the NADH-dependent formation of nitrite from nitrate. The standard 0.3-ml reaction mixture contained 80 mM Hepes-KOH (pH 7.5), 4.7 mM KNO_3, 4.7 mM EDTA, 16 μM Leupeptin, 10 μM FAD, 0.25 mM DTT, 5 μM Na-molybdate and 0.5 mM NADH as reductant. Reactions were initiated by the addition of extract (50 μl) and incubated for 5, 10 and 15 min at 30 °C. The reactions were determined by addition of 37 mM zinc acetate and 37 mM phenazine methosulfate (PMS). The formed nitrite was determined with a mixture of 1% sulfanilamide (in 2 N HCl) and 0.02% N-naphthylethylenediaminedihydrochloride and the concentration was measured via the absorbance at 540 nm.

Results

Protein contents in seeds in relation to the metabolite concentrations in the phloem sap and in whole leaves

A previous screening of seeds of 113 winter and 8 spring rape seed genotypes by near infrared reflectance spectroscopy (NIRS) revealed considerable differences in their protein (15–28%) and oil contents (38–56%; Möllers et al. 1996). The seeds of the winter rape seed breeding line DSV15 had a low protein content (about 19%) whereas the seeds of the spring rape seed cultivar Duplo had a medium protein content (about 25%). Further NIRS screening of *B. carinata* accessions identified one *B. carinata* accession (BRA1151/90) with a seed protein content of about 39% (Möllers et al. 1996). Although the seed oil and protein contents varied considerably depending on the growth conditions, the three selected genoytpes can be classified as having a comparatively low (DSV15), medium (Duplo) and high seed protein content (BRA1151/90; Table 1). Relative to the protein contents, the contents of free amino acid in the seeds were remarkably low (0.14–0.43% of DW) but higher in BRA1151/90 than in Duplo and DSV15 (Table 1).

The amino acid concentrations measured in the phloem of the three selected genotypes differed considerably (204–497 mM) and correlated positively with the seed protein contents (Table 1). Genotype BRA1151/90 not only showed the highest amino acid concentrations in the phloem but also the highest seed protein content. The amino acid concentrations in the phloem sap of Duplo and BRA1151/90 were 79% and 144% higher than in DSV15. The amino acid contents in the leaves of DSV15 and Duplo were similar, but 51% higher in BRA1151/90. The ratio of the total amino acid-to-sucrose concentration in the phloem was lowest in DSV15 (lowest seed protein content), followed by Duplo (medium seed protein content) and highest in BRA1151/90 (highest seed protein content). Generally, the ratio of the total amino acid-to-sucrose concentration was much higher in the leaves than in the phloem sap.

The level of the activity of NR, an enzyme which has a central role in the flow of nitrate into organic compounds, was also investigated. In-vitro assays of NR were carried out in leaf samples. The leaf NR activity was 35% higher in the *B. carinata* genotype BRA1151/90 than in the *B. napus* genotype Duplo and 50% higher than in DSV15.

Amino acid profiles of seed protein, phloem sap and whole leaves

Table 2 shows the amino acid compositions of seed protein, phloem sap and whole leaves of the three selected genotypes DSV15, Duplo and BRA1151/90. In the seeds the percentage of the sum of glutamate, glutamine and arginine relative to the total amino acid content increased slightly with the seed protein content. In the leaves and in the phloem sap the relative contents of most amino acids were similar, with glutamine and glutamate being predominant, followed by serine, threonine, and aspartate. These values concur with the results given by Weibull and Melin (1990). The glutamine content in the phloem and the leaves increased with

Table 1. Sucrose and amino acid concentrations (mM) and total amino acid-to-sucrose ratio of the phloem sap and the leaves of two selected *B. napus* genotypes and one *B. carinata* genotype differing in their seed protein and oil contents (% of DW). Samples were taken in the second half of the light period. Mean values ± SD from eight to ten (phloem sap) and four independent (leaves, seeds) measurements are given

Species Genotype	*B. napus* DSV 15	*B. napus* Duplo	*B. carinata* BRA 1151/90
Seeds			
Oil (% of DW)[a]	40–53	38–42	25–27
Protein (% of DW)[a]	15–23	22–28	37–41
Σ Amino acids (% of DW)	0.14 ± 0.02	0.18 ± 0.02	0.43 ± 0.03
Phloem sap of leaves			
Σ Amino acids (mM)	204 ± 85	365 ± 102	497 ± 53
Sucrose (mM)	978 ± 296	1384 ± 137	1351 ± 161
Σ Amino acids/Sucrose	0.21	0.26	0.37
Whole leaves			
Σ Amino acids [μmol (g FW)$^{-1}$]	9.0 ± 3.3	9.6 ± 0.7	13.6 ± 0.3
Sucrose [μmol (g FW)$^{-1}$]	6.6 ± 1.7	5.9 ± 1.4	8.4 ± 2.9
Σ Amino acids/Sucrose	1.4	1.6	1.6
Nitrate reductase activity [nmol nitrite (g FW)$^{-1}$ min^{-1}]	78 ± 15	87 ± 19	117 ± 24

[a]Data from Möllers et al. (1996)

Table 2. Content of each amino acid as a percentage of the total amino acid content in the seed proteins, the phloem sap and the leaves of two *B. napus* genotypes and one *B. carinata* genotype. Same samples as in Table 1. n.d., not determined; u.d.l., under detection limit

	Amino acid content (% of total amino acids)								
	Seeds (protein)			Phloem sap of leaves			Whole leaves		
	DSV15	Duplo	BRA1151	DSV15	Duplo	BRA1151	DSV15	Duplo	BRA1151
Aspartate + asparagine	10.9	10.3	9.9						
Aspartate				7.8	5.7	4.7	7.7	10.8	15.3
Asparagine				3.7	5.6	4.8	7.4	4.0	3.6
Glutamate + glutamine	18.9	19.8	22.6						
Glutamate				24.0	19.0	19.0	22.5	33.5	20.0
Glutamine				22.0	23.0	35.0	11.9	13.5	20.1
Serine	6.4	6.6	5.6	13.0	14.0	6.3	14.6	13.6	9.9
Threonine	8.0	6.6	5.2	6.4	6.3	6.7	7.3	4.2	6.2
Glycine	11.4	11.0	10.7	1.2	1.5	0.9	1.6	2.1	2.3
Alanine	9.3	9.5	8.5	4.0	3.1	8.6	1.8	1.2	2.6
Valine	6.3	6.3	5.6	3.2	4.1	3.8	4.1	4.3	5.0
Isoleucine	4.4	4.3	4.1	2.7	2.6	1.3	2.4	1.2	1.3
Leucine	8.3	8.5	8.3	1.3	3.3	1.0	2.4	0.7	1.3
Tyrosine	0.7	1.0	1.0	0.6	1.0	0.5	0.9	0.7	1.0
Phenylalanine	3.0	3.4	3.6	1.2	2.2	1.5	1.4	0.7	0.8
Tryptophane	n.d.	n.d.	n.d.	u.d.l.	0.4	u.d.l.	0.8	1.0	0.6
Arginine	3.6	4.0	7.2	2.9	2.9	1.8	7.8	4.1	6.9
Histidine	n.d.	n.d.	n.d.	1.3	u.d.l.	u.d.l.	2.4	3.2	2.3
Lysine	7.4	7.3	6.0	4.7	4.2	2.7	2.1	0.8	0.9
Methionine	1.4	1.5	1.7	0.7	0.6	0.7	0.5	0.5	0.3

the seed protein content of the respective genotype. The relative contents of arginine in the phloem sap and leaves were similar for all genotypes. The relative contents of glutamate and glutamine were lower in the seeds than in the phloem sap (Table 1), whereas the relative contents of glycine and leucine were higher.

Amino-N translocation in phloem

It should be noted that the phloem concentration of amino acids is not the only factor that determines the translocation rates of amino-N. However, it is possible to evaluate the translocation rates for amino acids from the ratio of the phloem concentrations of amino-N and sucrose and the translocation rate of total assimilated carbon. From the measurements of photosynthesis and the accumulation of photosynthetic products it can be deduced which portion of the total assimilates is translocated during the illumination period. In the *B. napus* line DSV15 about 1480 μmol C (g FW)$^{-1}$ was fixed during the 15-h light period, in Duplo about 1460 μmol C (g FW)$^{-1}$ and in the *B. carinata* line BRA1151/90 about 1620 μmol C (g FW)$^{-1}$ (Table 3). The difference in the carbon accumulation, as determined by the differences in the carbon contents of the major metabolites (starch, sucrose, hexoses, malate and amino acids), between the beginning and the end of the photoperiod, was 439 μmol C (g FW)$^{-1}$ in DSV15 leaves, 402 μmol C (g FW)$^{-1}$ in Duplo leaves, and 343 μmol C (g FW)$^{-1}$ in BRA1151/90 leaves. Thus, 1041 μmol C (g FW)$^{-1}$, 1058 μmol C (g FW)$^{-1}$, and 1277 μmol C (g FW)$^{-1}$, respectively, were maximally translocated during the light period (15 h) from the leaves of the *Brassica* genotypes. From the higher ratio of carbon to amino-N in the phloem of BRA1151/90, as compared to DSV15 and Duplo (Table 3), the conclusion can be drawn that the rate of amino-N translocation during the light period in BRA1151/90 [52 μmol N (g FW)$^{-1}$] is about 2-fold higher than in DSV15 and Duplo [DSV15: 24 μmol N (g FW)$^{-1}$; Duplo: 31 μmol N (g FW)$^{-1}$]. Hence, it follows that higher protein contents

Table 3. Assimilation, accumulation, and translocation of C, and translocation of N in *B. napus* (DSV15 and Duplo) and in *B. carinata* (BRA1151/90). The translocation rates of nitrogen were calculated from the carbon translocation rate and the amino-N to carbon (in sucrose and amino acids) ratio in the phloem sap. Mean values of six measurements of CO_2 assimilation and three for carbon assimilation ± SD are given

Species Genotype	*B. napus* DSV15	*B. napus* Duplo	*B. carinata* BRA1151/90
Net CO_2 assimilation [μmol C (g FW)$^{-1}$ (15 h)$^{-1}$]	1480 ± 310	1460 ± 240	1620 ± 250
Accumulated carbon [μmol C (g FW)$^{-1}$ (15 h)$^{-1}$]	439 ± 111	402 ± 95	343 ± 125
Translocated carbon [μmol C (g FW)$^{-1}$ (15 h)$^{-1}$]	1041	1058	1277
Σ Amino-N/Σ Carbon in phloem sap	0.023	0.029	0.041
Translocated nitrogen [μmol N (g FW)$^{-1}$ (15 h)$^{-1}$]	24	31	52

in the seeds correlate with higher amino-N translocation rates in the phloem.

Determination of the subcellular contents of sucrose and amino acids

The overall process of metabolite transfer from the leaf cells to the phloem is subjected to the concentration gradient between the cytosol of the source leaf cells and the phloem. In the present report these gradients of sucrose and amino acids have been evaluated from the analysis of subcellular contents in the source cells and the analysis of metabolite concentrations in the phloem sap. In order to analyze the distribution of sucrose and amino acids between the cytosolic and vacuolar compartments the method of non-aqueous fractionation was used (Gerhardt et al. 1987; Riens et al. 1991). Therefore, additional leaf samples were taken from the plants used for the analysis of the amino acid concentrations in the phloem and contents in the leaves. It should be noted that these analyses give an estimation of the subcellular contents because the evaluation is based on the simplification that the leaf consists of homogeneous cells. However, the mesophyll cells of leaves, i.e. spinach and potato, make up about 75% of the total leaf cells (Winter et al. 1994; Leidreiter et al. 1995). The cytosolic contents of sucrose and amino acids in both *B. napus* and *B. carinata* leaves were much higher than in the vacuole (Table 4). About 65% of the total amino acid and 60–75% of the total sucrose contents were found to be located in the cytosol, whereas the percentages in the vacuole were only 10% for total amino acids and 21–38% for sucrose. Smaller amounts of amino acids and sucrose were also found in the chloroplasts (data not shown). The osmotic balance between the cytosolic and vacuolar compartments is due to high contents of potassium, nitrate, sulfate, malate, and hexoses in the vacuolar compartment (data not shown). The amino acid content of the cytosol of *B. carinata* (BRA1151/90) was about 23% higher than in the *B. napus* line Duplo and 39% higher than in the line DSV15 (Table 4).

Table 4. Sucrose and total amino acid contents in the vacuole and in the cytosol of *B. napus* (DSV15 and Duplo) and *B. carinata* (BRA 1151/90) leaves. Samples were taken in the second half of the light period. Mean values ($n = 3$) ± SD are given

	Content [μmol (g FW)$^{-1}$]	
	Vacuole	Cytosol
B. napus (DSV15)		
Σ Amino acids	0.8 ± 0.3	6.2 ± 1.2
Sucrose	2.3 ± 1.1	3.7 ± 1.3
B. napus (Duplo)		
Σ Amino acids	1.4 ± 0.4	7.0 ± 0.6
Sucrose	2.1 ± 0.7	4.2 ± 0.7
B. carinata (BRA1151/90)		
Σ Amino acids	1.3 ± 0.4	8.6 ± 1.0
Sucrose	1.6 ± 0.5	5.7 ± 1.2

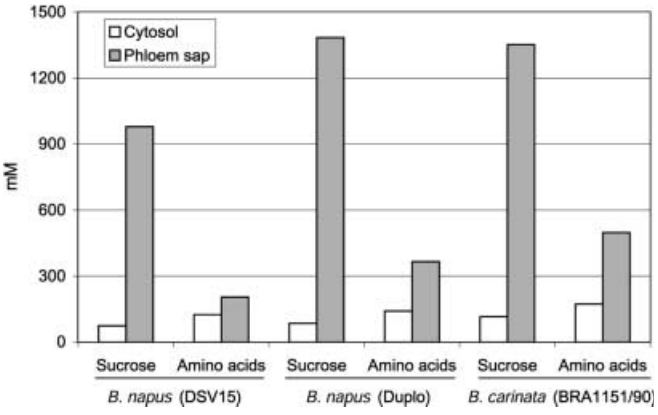

Fig. 1. Sucrose and amino acid concentrations in the cytosol of mesophyll cells and in the phloem of two *B. napus* lines (DSV15 and Duplo) and one *B. carinata* line (BRA1151/90)

Comparison of the concentrations of sucrose and amino acids in the cytosol of mesophyll cells and in the phloem sap

The conversion of subcellular contents based on fresh weight into concentrations requires knowledge of the size of the subcellular compartments. A direct determination can only be achieved by a laborious morphometric analysis of light and electron micrographs. However, as shown for barley, spinach, and potato the volume of the cytosolic compartment has been determined by means of morphometry as 4, 4, and 5% of the aqueous volume, respectively (Winter et al. 1993, 1994; Leidreiter et al. 1995). Assuming that the volume of the cytosolic compartment of *Brassica* leaves with 50 μl (g FW)$^{-1}$ is in the same range as in such different species as spinach [36 μl (g FW)$^{-1}$], barley [41 μl (g FW)$^{-1}$], and potato [55 μl (g FW)$^{-1}$], the cytosolic concentration of sucrose can be estimated from the cytosolic contents in Table 4 as 73 mM in *B. napus* (DSV15), 83 mM in *B. napus* (Duplo), and 114 mM in *B. carinata* (BRA1151/90), respectively. The corresponding amino acid concentrations are estimated as 124, 140 and 172 mM, respectively (Fig. 1). Thus the concentration gradient between the total amino acids in the phloem and in the cytosol of mesophyll cells is 1.6 in the *B. napus* line DSV15, 2.6 in Duplo and 2.9 in the *B. carinata* genotype BRA1151/90. The higher concentration gradients of amino acids correspond positively with the higher protein contents in the seeds of the latter cultivars. In all three genotypes the sucrose concentration in the phloem sap was about 12- to 17-fold higher than in the cytosol of the mesophyll cells.

Discussion

Influence of the amino acid transport on the protein content in seeds

The deposition of reserves in plant storage organs, such as seeds, is intimately linked to photosynthesis, nitrogen assimilation, nitrogen remobilization from senescing

Table 5. Amino acid concentrations in the phloem sap translocation rate of amino-N in the phloem, and protein contents in the seeds of different plant species

	Phloem sap amino acids [mM]	Phloem translocation rate of amino-N [μmol N (g FW)$^{-1}$ (15 h)$^{-1}$]	Seed protein [% of DW]
Zea mays (Illinois Low Protein)[a]	41	7.6	5
Zea mays (Illinois High Protein)[a]	118	24.9	23
B. napus (DSV15)	204	24	19
Pisum sativum ('Kleine Rheinländerin')	342		21
B. napus ('Duplo')	365	31	25
B. carinata (BRA 1151/90)	497	52	39

[a]Data from Lohaus et al. (1998)

tissue and long-distance transport of photoassimilates, such as sucrose and amino acids. In most plants, the transport of sucrose and amino acids from the site of biosynthesis in source leaves to the sink organs takes place in the phloem. Earlier observations with maize suggested that the pattern of substances translocated from the shoot to the developing seeds may affect the relative content of protein and carbon compounds in the seeds (Reggiani et al. 1985). In the present study the correlation between the nitrogen translocation in the phloem and the protein content in the seeds of different *Brassica* genotypes was analyzed. The amino acid concentration in the phloem sap of *B. napus* and *B. carinata* varied between 200 and 500 mM, which is exceptionally high (Table 1). Using the same method, amino acid concentrations of 40–200 mM were found in sugar beet, maize, barley and spinach (Riens et al. 1991; Winter et al. 1992; Winzer et al. 1996; Lohaus et al. 1998).

The amino acid concentration in the phloem sap is dependent on the external nitrate supply. Phloem sap from other *B. napus* lines (Bristol, Express Facon, Lirajet) grown on 4 mM nitrate contained between 350 and 700 mM total amino acids, whereas the same lines grown on 0.5 mM nitrate contained between 300 and 500 mM total amino acids (Z. Zhou and G. Lohaus, unpublished). Also under low nitrate conditions the amino acid concentration in the phloem sap of *B. napus* is much higher than in sugar beet, barley, maize or spinach, grown on higher external nitrate concentrations (4–7 mM; Riens et al. 1991; Winter et al. 1992; Winzer et al. 1996; Lohaus et al. 1998).

The amino acid concentrations in the phloem sap of different plant species correlate positively with their protein content in the seeds (Table 5). Of the two maize cultivars investigated Illinois High Protein maize and Illinois Low Protein maize, the former a cultivar with an especially high protein content in the seeds, also had the higher amino acid concentration in the phloem. The same correlation was observed here for the *B. carinata* line BRA1190/51 with the highest protein content in the seeds and the highest amino acid concentration in the phloem sap, as compared to the other *Brassica* species. The legume *Pisum sativum* showed a medium amino acid concentration in the phloem as well as a medium protein content in the seeds.

In *B. carinata*, the phloem translocation rate of amino-N during the light period [52 μmol N (g FW)$^{-1}$, Table 3] was considerably higher than that of all other plant species that we have analyzed so far [barley: 3.6 μmol N (g FW)$^{-1}$, spinach: 7.8 μmol N (g FW)$^{-1}$ (Riens et al. 1994), Illinois low- and high- protein maize: 7.6 and 24.9 μmol N (g FW)$^{-1}$ (Lohaus et al. 1998)]. Even in *B. napus* (DSV15 and Duplo), with protein contents in the seeds similar to Illinois high-protein maize (Table 5), the amino-N translocation rates [DSV15: 24 μmol N (g FW)$^{-1}$, Duplo: 31 μmol N (g FW)$^{-1}$] were nearly the same as in Illinois high-protein maize (Table 5).

Influence on the seed protein content of the concentration gradient of amino acids between the cytosol of mesophyll cells and the phloem

In apoplastic phloem loaders, such as *Brassica*, the amino acid transport into the phloem involves the passage of several membranes, at minimum the plasma membranes of the mesophyll cells and the phloem. To understand the transport process we determined the concentration gradients across these membranes. The total amino acid concentrations in the cytosol of mesophyll cells were evaluated as 124 mM in DSV15, 140 mM in Duplo, and 172 mM BRA1151/90, and the corresponding concentrations in the phloem as 204, 365, and 497 mM (Table 1, Fig. 1). The concentration gradient between the total amino acids in the phloem and in the cytosol of mesophyll cells was highest in BRA1151/90 (2.9) with the highest protein content in the seeds, followed by Duplo (2.6) with medium seed protein content and lowest in DSV15 (1.6) with the lowest protein content in the seeds. In other plant species, the concentration gradient of amino acids between the phloem and the cytosol of mesophyll cells was lower, i.e. 0.7 in barley (Lohaus et al. 1995) and 1.5 in maize (Lohaus et al. 1998).

Our earlier studies with different hybrids of fodder and sugar beet had shown that the amino acid contents in the leaves were similar, whereas the amino acid concentrations in the phloem sap were higher in those hybrids with higher amino acid contents in the taproots (Winzer et al. 1996). The results of the studies with and *B. napus* and *B. carinata* lines presented here concur with these earlier findings. Apparently, one major factor for the enhanced protein content in the seeds of *B. carinata* (BRA1151/90) and *B. napus* (Duplo) is the higher concentration gradient of amino acids between the

cytosol of mesophyll cells and the phloem, indicating that in BRA1151/90 and Duplo the phloem loading of amino acids is more effective than in DSV15. The uphill transport of amino acids from the mesophyll to the phloem is probably caused by proton-amino acid symporters (Kwart et al. 1993; Boorer and Fischer 1997). Different amino acid transporters, some of these exhibiting a broad substrate specificity, have been cloned by functional complementation of yeast mutants defective in amino acid transport by transformation with cDNA libraries from *Arabidopsis thaliana* (Kwart et al. 1993; Fischer et al. 1995). The similarity of the amino acid profiles in the leaves and phloem (Table 2) supports the idea that these amino acid transporters with a broad specificity are involved in phloem loading. Further studies on phloem loading of amino acids in different lines of *B. napus* will provide a better understanding of the regulation of the storage of protein in the seeds.

Other factors influencing the protein content of seeds

In addition to the extent of the concentration gradient of amino acids between the cytosol of mesophyll cells and the phloem sap, as well as the capacity for transport of organic nitrogen from the shoot to the developing seeds, other factors influence the relative content of protein in the seeds. Nitrate assimilation proceeds in plants not only in the leaves, but depending on the species and growth conditions, to a certain extent also in the roots. However, in temperate annual species with external nitrate concentrations in the range found in agriculture soils, nitrate assimilation of the shoots dominates that of the roots (Andrews 1986). Measurements of maize genotypes with low and high protein contents in the seeds showed that higher protein contents are correlated with higher NR activity and nitrate assimilation (Lohaus et al. 1998). The NR activity in leaves of the *B. carinata* line with high protein contents in seeds was also higher than in the *B. napus* lines with medium or low protein contents (Table 1). Moreover, a high protein content in seeds of high-protein varieties of rye or maize is related to an elevated asparagine synthesis (Dembinski and Bany 1991; Lohaus et al. 1998). Asparagine contributes up to 20% of the total amino acid content in the leaves and phloem sap of these varieties (Lohaus et al. 1998). By contrast, in the *B. napus* and *B. carinata* lines, asparagine represents only about 5% of the total amino acids in the leaves and in the phloem sap, and the percentage of asparagine was not correlated with the protein content in seeds (Table 2).

In most plant species a higher protein content in seeds is correlated with lower contents of carbon compounds like oil, starch or sugars (Table 1). Besides root and shoot metabolism, further metabolic properties in the seeds may influence the storage capacity of nitrogen or carbon compounds. To manipulate the quantity and quality of storage components in *B. napus* seeds, Kohno-Murase et al. (1994) constructed transgenic plants with an antisense gene for the storage protein napin in seeds. The seeds of the transgenic plants contained reduced amounts of napin, but the total protein contents of transgenic and wild-type seeds were similar, because the transgenic seeds accumulated more cruciferin (another storage protein) than control seeds.

Concluding remarks

The results described here demonstrate different positive correlations between phloem transport of amino acids and seed protein content: (i) the amino-N translocation in the phloem is higher in *Brassica* genotypes with higher seed protein contents, and (ii) the phloem loading process of amino acids, indicated by the concentration gradient of amino acids between the cytosol of source cells and the phloem, is more efficient in genotypes with higher seed protein content. With respect to the goals of breeding programs to reduce the protein content in seeds of *Brassica* and to increase the N-use efficiency, one strategy could be the reduction of the phloem transport of amino acids in *Brassica*. This strategy should be pursued in the future.

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