Introduction

An increasing body of evidence suggests that B might play a role in the structure and function of plant cell membranes. Further support comes from animal studies, where an essentiality of B for the embryogenic development was established (reviewed in Brown et al. 2002; Bolanos et al. 2004; Goldbach and Wimmer 2007). All currently known processes involving B are based on the ability of B to form reversible diester bonds with *cis*-diol containing molecules. We therefore hypothesize that B might play a direct structural role in membranes by forming cross-links with membrane-localized glycoproteins or glycolipids, thereby influencing the stability of the membrane or of specific membrane domains (Brown et al. 2002).

Since the isolation of intact B-complexes has proven to be difficult, likely because interactions of B with most known biological molecules are weak, we purified and identified membrane proteins capable of interacting with B by use of a combination of boronate affinity chromatography and proteomic analysis (Wimmer et al. 2009). In addition, we present evidence that B deficiency reduces the B-binding capacity of membrane proteins and seems to alter the protein spot pattern of 2D gels derived from membrane microdomains. We expect at least some of the identified proteins to be significant molecular partners of B *in vivo*, which may be useful in further characterization of B's roles in membrane structure and function.

Materials and Methods

Arabidopsis thaliana L. plants were grown in a hydroponic system in a climate chamber at day/night regimes of 20°C/18°C, 10h/14h light/dark and 50%/70% humidity. The nutrient solution contained 4 mM N, 1.5 mM K, 1.2 mM S, 2 mM Ca, 1 mM Mg, 1 mM P, 100 µM Fe, 30 µM B, 5 µM Mn, 1 µM Cu, 1 µM Zn and 0.7 µM Mo, and was adjusted to pH 5.5-6.0. Plants were harvested five weeks after germination. Boron deficiency treatments were implied during the last one to five days of growth by exchanging the nutrient solution with solution lacking B (< 1 µM B). *Zea mays* L. seeds were germinated between filter paper, supplied with one quarter strength nutrient solution, and harvested one week after germination.

Microsomal membrane fractions were extracted according to Sandelius and Morré (1990) with modifications. Plant roots were homogenized, cell debris removed, and a microsomal fraction was obtained by centrifugation at 90000 g (35 min). Membranes were solubilized by adding a final concentration of 1% (w/v) Triton X-100 (TX100).

A two-phase partitioning system (PEG 3500/dextrane T500, 6.2% each) was used to enrich plasma membranes (Larsson et al. 1987). After solubilization in 1% TX100, microdomains were purified by centrifugation at 250000 g for 20h in a sucrose density gradient (15-45% w/w) (Peskan et al. 2000) The microdomain fraction visible as light-scattering band was collected, pelleted and resuspended in lysis buffer for 2-DE.

Proteins with the ability to bind to B were purified using agarose-bound phenylboronate based on the method of Middle et al. (1983) with some modifications. Samples were equilibrated with buffer (50 mM taurine/NaOH, 3 mM $MgCl_2$, pH 8.7), incubated with pre-washed resin, and the non-binding fraction collected by centrifugation (10 s, 500 g). The resin was then thoroughly washed, and the bound fraction eluted with buffer containing 50 mM sorbitol.

All fractions were precipitated over night with acetone/DTT at -20°C. Pellets were resuspended in buffer (63 mM Tris, 10% glycerol, 2% SDS, 5% mercaptoethanol, 0.05% bromophenol blue, pH 6.8) for 1D SDS PAGE and in lysis buffer (7 M urea, 2 M thiourea, 0.5% TX-100, 20 mM DTT, 5 mM Pefabloc (Fluka), 20 mM Tris base, 0.2% pharmalytes pH 3-10) for 2-DE, which was carried out using 11 cm pH 3-10 IPG strips and an IPGPhor Iso-

electric Focusing System (Amersham Biosciences, Freiburg). The second dimension was carried on 12.5% polyacrylamide gels. For protein identification, spots were excised, digested with trypsin, and analyzed using an Ultraflex I MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). Proteins were identified by Mascot searches (version 2.2.04, Matrix Science, London, United Kingdom) using locally installed MSDB or NCBInr databases. Proteins were considered as matches only if they had a significant Mascot score and corresponding theoretical and apparent molecular weights after SDS-PAGE.

Results and discussion

B-binding proteins from root microsomal preparations

We were able to separate two protein fractions, one considered to be B-binding and the other non B-binding, from *A. thaliana* and *Z. mays* roots. Spot patterns consistently obtained in several independent experiments following 2-DE are shown in Fig. 1. Sixteen (*A. thalania*) and eleven spots (*Z. mays*), respectively, from the B-binding fraction were identified by MALDI-TOF peptide mass fingerprinting (Tab. 1).

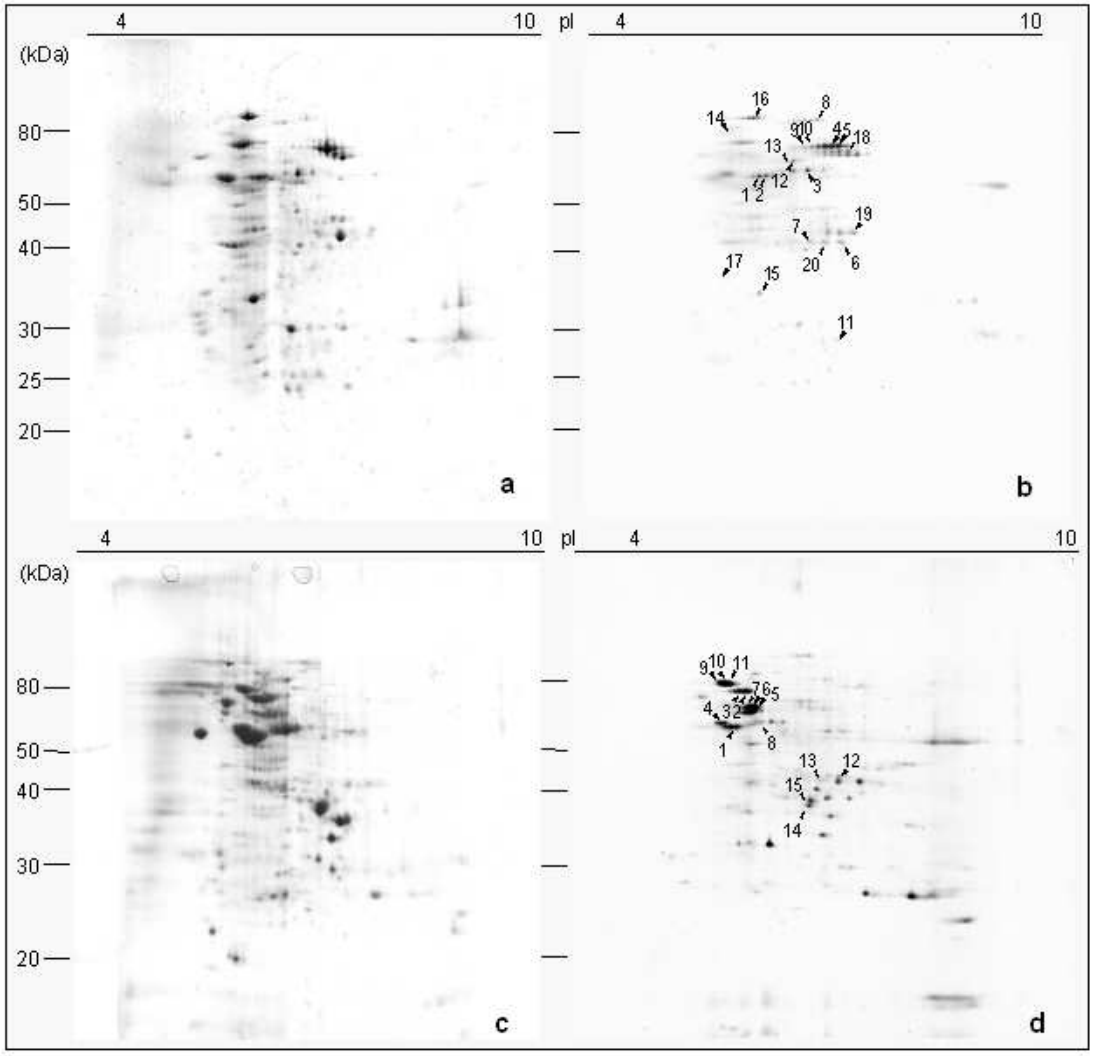

Figure 1: 2-D gels of non-B-binding (a,c) and B-binding (b,d) fractions of *A. thaliana* (a,b) and *Z. mays* (c,d) root microsomal fractions. Arrows and numbers in (b,d) correspond to numbers of identified spots in Table 1.

Table 1: B-binding proteins identified in *Arabidopsis thaliana* and *Zea mays* root microsomal preparations; spot No. corresponds to the numbers indicated in Fig. 1b and d.

No.	Protein name	Gene name	Mascot score	seq. cov.	MW_{th} [a] (kD)	MW_{obs} [b] (kD)
Arabidopsis thaliana						
identified:						
1, 2	ATP synthase subunit beta (mitoch.)	At5g08670	111	47%	59.6	50-60
3	ATP synthase subunit alpha, (mitoch.)	AtMg01190	78	31%	55.0	50-60
4	thioglucosidase 3D precursor	pyk10	60	22%	59.7	60-70
5	beta-glucosidase	psr3.1	107	36%	60.0	60-70
6	glyceraldahyde-3-phosphate dehydrogenase	At3g04120	80	44%	37.0	ca. 40
7	fructose bisphosphate aldolase-like protein	At3g52930	105	48%	38.5	ca. 40
11	carbonic anhydrase homolog	At1g70410	93	41%	28.4	30-40
16	jasmonate-inducible protein isolog (put. lectin protein, myrosinase binding protein-like; similar to MBP1)	At3g16460	135	40%	72.4	80-90
identified, but not always present						
8	cobalamin-indep. methionine synthase	At5g17920	56	21%	84.3	80-90
9, 10	NADP dep. malic enzyme-like protein	At5g11670	107	35%	64.4	60-70
12, 13	prob. mitoch. processing peptidase subunit beta	At3g02090	112	45%	59.1	ca. 60
14	luminal binding protein BiP2	At5g28540	88	30%	73.5	ca. 80
15	jasmonate inducible protein isolog (myrosinase binding protein-like, put. lectin)	At3g16420	109	68%	32.1	30-40
putative, but highly regularly present						
17	endomembrane-associated protein	At4g20260	58	58%	24.6	30-40
18	putative beta-glucosidase	Bglu22	39	25%	60.0	60-70
19	putative myrosinase-associated protein	At1g54000	35	41%	43.1	30-40
20	unknown (possibly peroxidase 39)	At4g11290	39	41%	35.6	40-50
Zea mays						
identified:						
1	mitochondrial ATP synthase subunit beta	ATPB	85	25%	59.2	50-60
2, 3	vacuolar H+-ATPase catalytic subunit	PcVHA-A1	92	18%	69.0	70-80
4	vacuolar ATPase B subunit	Q7FV25_ORYSA	43	6%	54.1	50-60
5, 6	beta-glucosidase	Glu1	134	32%	64.5	60-70
7	beta-glucosidase	Glu1 or Glu2	118	23%	64.5	60-70
8	probable UDPglucose-6-dehydrogenase	UGDH_SOYBN	63	17%	53.5	50-60
9, 10	luminal-binding protein 2 (cBiPe2)	BIP2_MAIZE	182	30%	73.2	70-90
11	luminal-binding protein 3 (cBiPe3)	BIP3_MAIZE	82	19%	73.3	70-90
putative:						
12, 13	fructose-bisphosphate aldolase, cytosolic	ALF_MAIZE	59	17%	39.0	ca. 40
14, 15	Retrotransposon protein, put., unclassified	Os11g22130	56	20%	31.3	30-40

[a] theoretical molecular mass determined from database
[b] approximate molecular mass observed in the gel

The B-binding proteins common to both *A. thaliana* and *Z. mays*, were mitochondrial ATP synthase subunit beta, beta-glucosidases, luminal binding protein and fructose bisphosphate aldolase (Tab. 1). Additional spots were detected after silver staining of replicate gels, but the abundance of these proteins was too low for subsequent analysis.

Among the consistently most prominent B-binding proteins in both plant species were beta-glucosidases. They were always present as a series of spots in close proximity (Fig. 1b, d), which is typical for protein isoforms with different co- or post-translational modifications. In addition, they were also visible in the non-B-binding protein fraction (Fig.

1a, c), indicating that some isoforms are B-binding, and some are not. Since several sugars are known to easily form di-esters with B, it is likely that the B-binding isoforms harbour glycosyl residues, and myrosinases, a subgroup of beta-glucosidases, are known to be glycosylated in dicot plant species (Morant et al. 2008).

The strong B-binding capacity of ATPase subunits is interesting, since B supply has been shown earlier to affect membrane H^+ transport (Ferrol et al. 1993) and the activity of ATPase in lily pollen grains (Obermeyer et al. 1996).

B-binding membrane proteins are released from the membrane under B deficiency

In order to determine whether B-binding proteins are integral membrane components, we compared the protein pattern of membranes solubilized with (+TX) or without (-TX) detergent (Fig. 2). When *A. thaliana* plants were grown for 5 weeks with sufficient B supply (30 µM B), detergent was necessary to solubilize most of the B-binding proteins. However, when subjected to 5 d of B deficiency just before harvest, some B-binding proteins became soluble without detergent addition (Fig. 2b), indicating that they were no longer tightly embedded in the membrane. Even though the underlying mechanism is still obscure, it might be speculated that B-linkage is involved in the membrane-binding of glycoproteins, and that an erroneous glycosylation under B deficiency prevents membrane attachment. An analogous effect was observed in B deficient pea nodules, where lack of a specific carbohydrate epitope of a glycoprotein prohibited its incorporation into peribacteriod membranes (Bolanos et al. 2001).

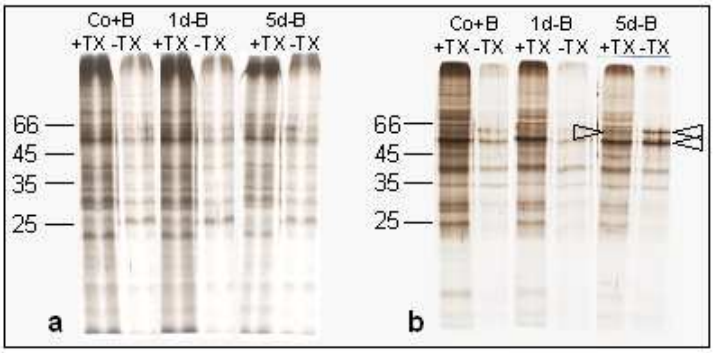

Figure 2: 1-D gels of non-B-binding (a) and B-binding (b) fractions of *A. thaliana* root microsomal preparations after solubilization with (+TX) and without (-TX) Triton-X100. Plants were grown either with sufficient (Co, 30 µM B) or for 1 to 5 d with deficient (<1 µM B) B supply. Arrows in (b) indicate significant changes in protein pattern after 5 d of B deficiency.

B-binding capacity of membrane proteins is reduced under B deficiency

When microsomal preparations from *A. thaliana* roots containing the same amount of protein were solubilized, subjected to boronate affinity chromatography and resolved by 2-DE, the abundance and distribution of the non-B-binding proteins were similar irrespective of B supply (Fig. 4a-c). However, overall abundance of B-binding proteins was clearly reduced after 4 d (not shown) and 5 d of B deficiency (Fig. 4d-f). Although an effect on gene expression and/or translation cannot be ruled out, it seems more likely that changes occurred in post-translational glycosylation. Small changes in the glycosylation pattern of molecules can greatly affect their B-binding capacity, as was shown in the *A. thaliana* mur1-1 mutant, where substitution of fucose residues by galactose resulted in greatly reduced formation of dimeric RGII and reduced growth (O'Neill et al. 2001).

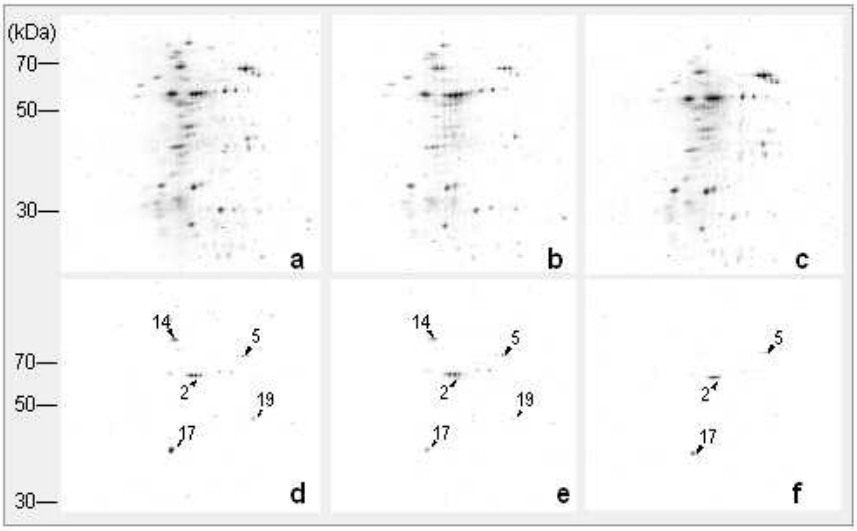

Figure 3: 2-D gels of non-B-binding (a-c) and B-binding (d-f) fractions of solubilized *A. thaliana* root microsomal preparations. Initial sample volumes contained the same amount of protein. Plants were grown either with sufficient (30 µM B) (a,d), or for 1 d (b,e) and 5 d (c,f) with deficient B supply (<1µM B in nutrient solution). Arrows and numbers in Fig. 4d-f correspond to those shown in Tab. 1.

Is B involved in the formation or stabilization of membrane microdomains?

We hypothesize that crosslinking of glycoproteins could be involved in the formation of membrane microdomains, or the insertion of certain proteins into such domains. Microdomains have been implicated in root hair and pollen tube growth (Kost et al. 1999), as well as in animal processes characterized by rapid membrane development (Simons and Ikonen 1997), all of which are especially prone to B deficiency. Since B-binding ATP synthase alpha and beta subunit, vacuolar ATPase B subunit, beta-glucosidases, and jacalin lectin family proteins (Tab. 1) have been previously described as components of membrane rafts (Bhat and Panstruga 2005; Borner et al. 2005; Morel et al. 2006), it is tempting to speculate that B might indeed have a function in anchoring these proteins in membrane microdomains. If this was true, lack of B should be expected to result in changes of the protein composition of microdomains. Our first results indicate that such changes can indeed be observed (Fig. 4).

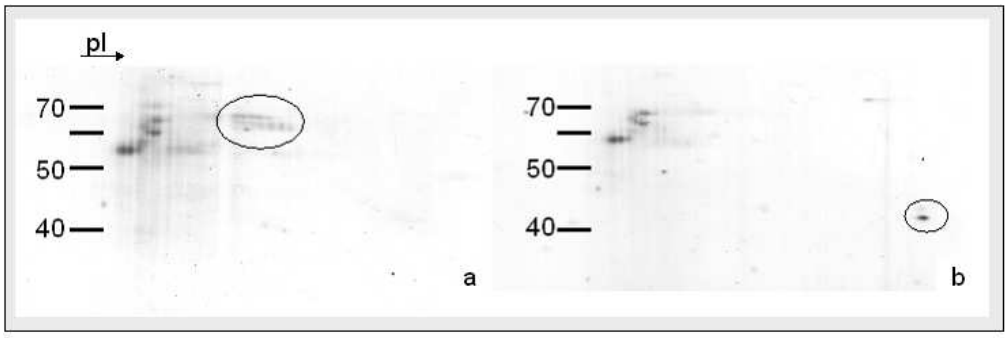

Fig. 4: 2D gels of microdomains isolated from *A. thaliana* leaves; plants were grown with sufficient (30 µM B; gel a) or deficient (5 µM B; gel b) B supply; circles mark areas clearly different between treatments.

Even though still a matter of speculation, such a role of B in plant cell membranes would be in line with the relatively large number of identified B-binding membrane proteins and would offer a mechanistic explanation for the multitude of observed B-dependent processes.

Conclusions

The fact that a range of B-binding membrane proteins has been identified does not *per se* demonstrate any physiological relevance. It is, however, one of the premises required for any function of B at membranes to occur. So far, all our results are in line with the hypothesis that B indeed has a function in the stabilization of membranes or membrane domains.

Acknowledgements

This work was funded by a grant of the Deutsche Forschungsgemeinschaft (DFG).

References

Bhat, R.A. and Panstruga, R. (2005) Lipid rafts in plants. *Planta* 223: 5-19.

Bolanos, L., Cebrian, A., Redondo-Nieto, M., Rivilla, R. and Bonilla, I. (2001) Lectin-like glycoprotein PsNLEC-1 is not correctly glycosylated and targeted in boron deficient pea nodules. *Mol. Plant Microbe In.* 14: 663-670.

Borner, G.H.H., Sherrier, D.J., Weimar, T., Michaelson, L.V., Hawkins, N.D., MacAskill, A. et al. (2005) Analysis of detergent-resistant membranes in Arabidopsis. Evidence for plasma membrane lipid rafts. *Plant Physiol.* 137: 104-116.

Brown, P.H., Bellaloui, N., Wimmer, M.A., Bassil, E.S., Ruiz, J., Hu, H. et al. (2002) Boron in plant biology. *Plant Biol.* 4: 205-223.

Ferrol, N., Belver, A., Roldan, M., Rodriguezrosales, M.P., Donaire, J.P. (1993) Effects of boron on proton transport and membrane properties of sunflower (*Helianthus Annuus* L.) cell microsomes. *Plant Physiol.* 103: 763-769.

Goldbach, H.E. and Wimmer, M.A. (2007) Boron in plants and animals: Is there a role beyond cell-wall structure? *J. Plant Nutr. Soil Sci.* 170: 39-48.

Kost, B., Lemichez, E., Spielhofer, P., Hong, Y., Tolias, K., Carpenter, C. et al. (1999) Rac homologues and compartmentalized phosphatidylinositol 4,5-bisphosphate act in a common pathway to regulate polar pollen tube growth. *J. Cell Biol.* 145: 317-330.

Larsson, C., Widell, S. and Kjellborn, P. (1987) Preparation of high purity plasma membranes. *Meth. Enzymol.* 148: 558-568.

Middle, F.A., Bannister, A., Bellingham, A.J. and Dean, P.D.G. (1983) Separation of glycosylated haemoglobins using immobilized phenylboronic acid. *Biochem. J.* 209: 771-779.

Morant, A.V., Jorgensen, K., Jorgensen, C., Paquette, S.M., Sanchez-Perez, R., Moller, B.L. et al. (2008) Beta-glucosidases as detonators of plant chemical defense. *Phytochem.* 69: 1795-1813.

Morel, J., Claverol, S., Mongrand, S., Furt, F., Fromentin, J., Bessoule, J.J. et al. (2006) Proteomics of plant detergent-resistant membranes. *Mol. Cel. Proteomics* 5: 1396-1411.

Obermeyer, G., Kriechbaumer, R., Strasser, D., Maschessnig, A. and Bentrup, F.W. (1996) Boric acid stimulates the plasma membrane H^+-ATPase of ungerminated lily pollen grains. *Physiol. Plant.* 98: 281-290.

O'Neill, M., Eberhard, S., Albersheim, P. and Darvill, A. (2001) Requirement of borate cross-linking of cell wall rhamnogalacturonan II for Arabidopsis growth. *Science* 294: 846-849.

Peskan, T., Westermann, M. and Oelmueller, R. (2000) Identification of low-density Triton X-100-insoluble plasma membrane microdomains in higher plants. *Eur. J. Biochem*. 267: 6989-6995.

Sandelius, A.S. and Morre, D.J. (1990) Plasma membrane isolation. In *The plant plasma membrane - structure, function and molecular biology.* Edited by Larsson, C. and Moeller, I.M. pp. 44-70. Springer Verlag, Berlin.

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature* 387: 569-572.

Wimmer, M.A., Lochnit, G., Bassil, E., Mühling, K.H., Brown, P.H. and Goldbach, H.E. (2009) Membrane-associated, boron interacting proteins isolated by boronate affinity chromatography. *Plant Cell Physiology* 2009, doi: 10.1093/pcp/pcp073.