Transport and Assimilation

THE IMMUNOPHILIN-LIKE *TWISTED DWARF* PROTEIN INTERACTS WITH THE C-TERMINI OF *ARABIDOPSIS* ABC TRANSPORTERS *IN VITRO*

M. Geisler[2], Ü. Kolukisaoglu[1], B. Schulz[1] and E. Martinoia

Université de Neuchâtel, Physiologie Végétale, Switzerland and [1]Botanisches Institut der Universität zu Köln, Germany ([2] e-mail: markus.geisler@bota.unine.ch)

The *Arabidopsis* twisted dwarf (TWD) mutant has been isolated from a T-DNA tagged *Arabidopsis* population and analyzed on the morphological and molecular level. Mutant plants show an elongated life cycle and a drastic pleiotropic phenotype due to a reduction of cell elongation and a desorientated (twisted) growth. The mutation was shown to be caused by a disruption of an immunophiline-like gene which belongs to the class of the FK506 binding (FKBP) immunophilins. In higher plants, immunophilins recently have been identified in various tissues and cellular compartments [1]. A Mutation in the *Arabidopsis* PASTICCIN01 gene encoding a FK506-binding protein-like protein has also a dramatic effect on plant development [2].

The Arabidopsis TWD protein binds calmodulin - A putative calmodulin (CaM)-binding domain has been localized in the C-terminus (aa 310 - 326) of the TWD upon comparison with motifs of different origin that are known to be responsible for CaM binding [3]. The CaM-binding domain was demonstrated by CaM overlays using biotinylated CaM. Both the full-length enzyme (TWD4) and the full-length enzyme minus a putative C-terminal transmembrane domain (TWD3) purified by Ni-NTA agarose affinity protein chromatography were able to bind CaM in a calcium-dependent manner as described for other plant immunophilins [4].

The TWD protein binds to the C-termini of ABC transporters in the absence of an immunosupressive compound - In analogy to animal and yeast systems we assumed that also the TWD protein interacts with other cellular protein partners. As shown by yeast 2-hybrid experiments, beside hsp90 - a well-known interacting partner of immunophilins [1] - interestingly the C-terminal ends of *Arabidopsis* ABC transporters [7] - two different MDR- and two MRP-like clones, respectively - were found to be often associated with the TWD protein

Ni-affinity purified TWD3 and TWD4 were used to synthesize a TWD affinity matrix by immobilizing them to affigel beads. Matrix-bound TWD were incubated with cleared *E. coli* supernatants from cells overproducing the C-terminal ends of the ABC transporters shown to interact in the yeast 2-hybrid experiments. The C-termini of the two MRPs At-MRP1 and At-MRP7 and the MDR At-PGP1 [6] were selectively bound by both TWD3 and TWD4. The *Arabidopsis* TWD-ABC transporter complex was stable in the absence of an immunosuppressive compound. A similar result has been reported for the yeast FKBP12-calcineurin complex but in contradiction to our data, in the yeast system FK506 leads to a dramatic increase of the interaction [5].

Currently, we are investigating the effect of CaM on the formation of the ABC-TWD complexes. First experiments revealed that binding to the C-terminus of At-PGP1 seems to be inhibited in the presence of CaM; inhibition is found in the presence but not in the absence of Ca^{2+}. In contrast, CaM had little or no influence on the complex formation between TWD and the C-termini of the two MRPs.

Current work - We are currently quantifying the complex formation by surface plasmon resonance (SPR) spectroscopy using a BIAcore 1000. In initial qualitative experiments all three purified ABC transporter C-termini were specifically retained by the TWD3 protein coupled to a CM5 sensor chip via amino coupling. In contrast to the two MRPs, only PGP1 could be completely removed from the immobilized TWD by 2 M NaCl, indicating a mainly electrostatic type of interaction of the latter.

The intracellular localization of the TWD and both interacting MRPs is unknown while for the interacting At-PGP1 a plasma membrane localization has been postulated [6]. As two putative interacting membrane proteins should residue in the same intracellular compartment, we are currently (co)immunolocalizing interacting partners in microsomal fractions using immunological and immunoprecipitation techniques. First immunological data confirm a plasma membrane origin for At-PGP1 while the TWD seems to reside in different - if not all - membrane compartments.

In summary, our results provide genetical and biochemical evidence that the TWD protein and *Arabidopsis* MDR- and MRP-like transporters are indeed interacting partners. Our data point to the fact that MDRs employ different binding sites and/or binding mechanisms on the TWD protein compared to MRPs. On the other hand Ca^{2+}/CaM might be a regulatory factor that could be responsible for selective binding of TWD to ABC transporter subclasses.

According to the regulatory nature of immunophilins one can expect first insights into higher plant ABC transporter regulation. In addition, due to the crucial phenotype of the TWD mutants, a detailed knowledge of ABC transporter regulation may give further insights in processes involved in plant development.

References :

[1] Reddy, R., Kurek, I., Silverstein, A.M., Chinkers, M., Breiman, A., and Krishna, P. (1998) Plant Physiol. 118:1395

[2] Vittorioso, P., Cowling, R., Faure, J., Caboche, M. and Bellini, C. (1998) Mol. Cell. Biol. 18: 3034

[3] Geisler, M., Axelsen, K., Harper, J.F., and Palmgren, M.G. (2000). Biochim. Biophys. Acta (in press)

[4] Hueros, G., Rahfeld, J. Salamini, F. and Thompson, R. (1998) Planta 205: 121

[5] Cardenas, M.E., Hemenway, C., Muir, R.C., Ye, R., Fiorentino, D, and Heitmann, J (1994) EMBO, 13: 5944

[6] Sidler, M., Hassa, P., Hasan, S., Ringli, C., and Dudler, R. (1998) Plant Cell, 10: 1623

[7] Tommasini, R., Vogt, E., Fromenteau, M., Hortensteiner, S., Matile, P., Amrhein, N., and Martinoia, E. (1998)Plant J. 13: 773

ASSIMILATORY SULFATE REDUCTION IN C_3, C_3-C_4, AND C_4 SPECIES OF *FLAVERIA.*

Anna Koprivova, Michael Melzer§, Peter von Ballmoos, Therese Mandel, Cris Kuhlemeier, Christian Brunold, and Stanislav Kopriva

Institute of Plant Physiology, Altenbergrain 21, 3013 Bern, and § Institute of Plant Genetics and Crop Plant Research, Corrensstrasse 3, 06466 Gatersleben, Germany

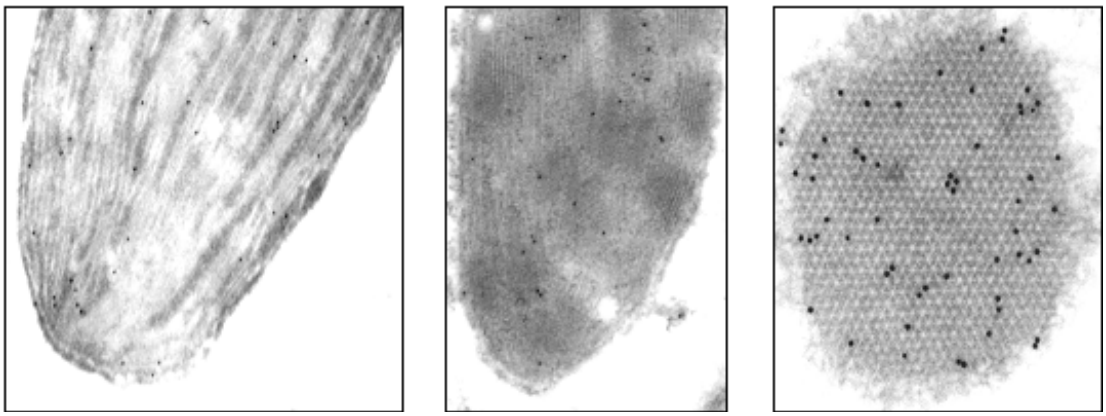
Assimilatory sulfate reduction is a pathway used by procaryots, fungi, and photosynthetic organisms to convert inorganic sulfate to sulfide, which is further incorporated into carbon skeleton of amino acids to form cysteine or homocysteine. The key step in this pathway is the reduction of activated sulfate, adenosine 5´-phosphosulfate (APS) by APS reductase (APR) [1]. It is generally accepted that in C_4 plants the first steps of sulfate assimilation catalyzed by ATP sulfurylase and APR are confined to bundle sheath cells [2, 3]. In order to determine whether this is a pre-requisite or a consequence of C_4 photosynthetic mechanism we analyzed the activity and expression of these two enzymes in C_3, C_3-C_4, C_4-like, and C_4 species of the genus *Flaveria*.

In a first step, activities of ATP sulfurylase and APR were measured in young leaves of 8 species with different photosynthesis types The APR activity in C_4 species *F. palmeri*, *F. trinervia*, and *F. australasica* was significantly higher than in C_3 and C_3-C_4 intermediate species. ATPS activities showed a similar pattern, with the highest activity in *F. palmeri.* Cysteine and glutathione levels were measured in these species and the results corresponded to the distribution of APR activity, i.e. in C_4 and C_4-like species the GSH and Cys concentrations were higher than in C_3 and C_3-C_4 species. Analysis of mRNA levels, and protein accumulation revealed that the enzyme activity correlated with the mRNA and protein levels. Therefore, we conclude that the average APR activity increases with the evolution towards C_4 photosynthesis.

Partial cDNAs for ATPS and APR were cloned from *F. trinervia* by RT-PCR with degenerate primers against conserved domains.. These cDNAs were used as probes for *in situ* RNA hybridization in 6 *Flaveria* species. As controls, *in situ* hybridizations with cDNAs for Rubisco small subunit, PEPCase, and H-protein of glycine decarboxylase showed expected patterns of differential mRNA expression, however, ATPS and APR mRNA were localized in both mesophyll and bundle-sheath cells of C_4-like and C_4 species. This result was confirmed by Northern analysis with RNA isolated from mesophyll and bundle-sheath cells of two C_4 species, the mRNAs for ATPS and APR were again present in both cell types at about the same levels.

Immunolocalization experiments with monospecific antibodies against APR2 from *A. thaliana* confirmed the presence of APR protein in both cell types. APR was localized to chloroplasts in both mesophyll and bundle-sheath cells of *F. trinervia* and, surprisingly, to peroxisomes in all species analyzed (Figure 1).

We conclude, that the previously reported exclusive localization of assimilatory sulfate reduction to bundle-sheath cells of C_4 plants was due to the limitation of the analysis to monocot species only. We also hypothesize that in peroxisomes APS reductase is active as dehydroascorbate reductase [4] in a defence against reactive oxygene species.



bundle-sheath chloroplast mesophyll chl. peroxisom

Figure 1. Immunolocalization of APR in leaves of C_4 dicot *F. trinervia*.

References

Brunold, C. (1993). In Sulfur nutrition and sulfur assimilation in higher plants, L.J. De Kok et al., eds (The Hague, The Netherlands: SPB Academic Publishing), pp. 61-75.

Gerwick, B. C., Ku, S. B., and Black, C. C. (1980) *Science* 209, 513-515

Schmutz, D. and Brunold, C. (1984) *Plant Physiol.* 74, 866-870

Bick, J.-A., Åslund, F., Chen, Y. and Leustek, T. (1998) *Proc. Natl. Acad. Sci. USA* 95, 8404-8409.

CHARACTERIZATION OF TWO FAMILIES OF PHOSPHATE TRANSPORTERS FROM VASCULAR PLANTS

Pierre Daram, Silvia Brunner, Christine Rausch, Cyrill Steiner, Nikolaus Amrhein and Marcel Bucher

Institute of Plant Sciences, Experimental Station Eschikon, ETH Zurich

marcel.bucher@ipw.biol.ethz.ch

Our major interest is the acquisition of phosphate (Pi) at the root/soil interface and its subsequent transport within the plant. We currently characterize members of two distinct families of Pi transporters from vascular plants, named Pht1 and Pht2. Pht1 transporters are predominantly expressed in roots and their expression is Pi repressed. Members of the Pht2 family differ from those of the Pht1 family in primary structure, affinity for Pi and function. Whereas Pht1 proteins exhibit high affinity for Pi transport, Pht2 from Arabidopsis is a low-affinity transporter. Its expression is confined to the shoot and is not Pi repressible. The generation of transgenic plants with altered Pht2 expression levels will give more information about the role of Pht2 transporters in phosphorus homeostasis.

Daram P, Brunner S, Persson BL, Amrhein N and Bucher M (1998) Functional analysis and cell-specific expression of a phosphate transporter from tomato. Planta, 206: 225-233

Daram P, Brunner S, Rausch C, Steiner C, Amrhein N and Bucher M (1999) *Pht2;1* encodes a low-affinity phosphate transporter from *Arabidopsis thaliana*. Plant Cell, 11**:** 2153-2166

TARGETING OF TWO DIFFERENT GFPS TO DIFFERENT VACUOLES IN PLANTS

Gian-Pietro Di Sansebastiano, Nadine Paris, Ricardo Flückiger, Sophie Marc-Martin and Jean-Marc Neuhaus

Laboratoire de Biochimie, Université de Neuchâtel, rue Emile-Argand 9, 2007 Neuchâtel

e-mail: Jean-Marc.Neuhaus@bota.unine.ch

An individual plant cell may contain at least two functionally and structurally distinct types of vacuoles: protein storage vacuoles and lytic vacuoles. Presumably, a cell storing proteins in vacuoles must maintain these compartments separate to prevent exposure of the storage proteins to the acidified environment of a lytic vacuole where they would be degraded by active hydrolytic enzymes. Plant cells must generate these two types of vacuoles, maintain them as separate organelles, and direct soluble proteins from the secretory pathway specifically to one or the other via separate transport pathways.

Analysis of several precursors of vacuolar proteins led to the identification of three classes of vacuolar sorting determinants (VSD) (1): 1) Sequence-specific VSDs, often found within N-terminal propeptides and containing an essential Ile (or Leu). They can interact in vitro and in vivo with a member of a family of putative vacuolar sorting receptors (VSR) originally described as BP-80, which was isolated from clathrin-coated vesicles from immature pea cotyledons (2). 2) C-terminal VSDs with little sequence conservation but which can be blocked by addition of C-terminal Gly. No receptor is known but transport of such proteins to vacuoles is more sensitive than those from the first class to wortmannin, a phosphatidylinositol-3-kinase inhibitor, and 3) Internal VSDs are less well characterized and appear to cause aggregation. Proteins of this class leave the Golgi in smooth dense vesicles and accumulate in protein bodies.

While the coexistence in single cells of vacuoles with diffent contents was known to microscopists, biochemical studies relied on extracts and fractions which prevented the distinction of these vacuoles. Only recently, with the help of antibodies specific to various soluble and membrane proteins it became possible to address the function of different vacuoles in single cells. In pea and barley root cells, the tonoplat intrinsic proteins ?-, ?- and ?-TIP can be found in different small compartments, but sometimes two or even three TIPs have been found in the same structures. Similarly, barley aleurain and lectin have been found in separate compartments, but also together (3, 4). However, these results are limited to a few cell types that have been fixed for immunostaining.

In order to study the trafficking of vacuolar proteins in living cells, we have developped two different forms of the Green Fluorescent Protein (GFP) fused to either of two VSDs: the N-terminal propeptide of barley aleurain, which contains a sequence-specific VSD, for AGFP, or the C-terminal propeptide of tobacco chitinase I, which is a C-terminal VSD, for GFPT (5, 6).

Expression of these two GFPs in tobacco leaf protoplasts, a mixture of different cells differing in size and number of chloroplasts, revealed the existence of two different vacuolar compartments, with different size distributions in the different cell types. In the large, chloroplast-rich protoplasts approximately three quarters of the large central vacuoles accumulated the GFPT, while a quarter accumulated AGFP. In protoplasts with few chloroplasts GFPT was found in about 8% of the central vacuoles, and AGFP in about 15%. These localizations correlated with the pH of the central vacuole as revealed by Neutral Red accumulation: neutral for GFPT and acidic for AGFP. When the GFPs were not found in the central vacuoles, they were found in smaller compartments which differed in appearance for the two GFPs: about the size of chloroplasts for GFPT but much smaller for AGFP. In lytic vacuoles GFP appeared much less stable. Fluorescence was weak and disappeared rapidly after synthesis was stopped.

Evacuolation of mesophyll protoplasts expressing either GFP allowed to observe the regeneration of the central vacuole. The marker for the acidic vacuole was observed in the central vacuole as soon as it became visible. In contrast, the marker for neutral vacuoles, GFPT, was never observed in the newly formed vacuole within the first 36 hours of regeneration, but remained confined to the peripheral compartments described before. Only after more than two days, the GFPT appeared in the central vacuole, probably by fusion of the two types of vacuoles.

Transgenic plants were also produced. In tobacco, only plants expressing GFPT were obtained. These plants lacked any green fluorescence, except for the stomates, where a small round vacuole was fluorescent, and for a few cells in roots and hypocotyl. Interestingly, digestion of leaves produced highly fluorescent protoplasts. It is possible that in tobacco most cells harbour only hybrid vacuoles, where GFP is degraded by digestive enzymes. This would explain why marker proteins (barley lectin and sweet potato sporamin) were observed in the same vacuoles in transgenic tobacco (7). Transgenic Arabidopsis were also produced. Both GFPs were visible but in different tissues. AGFP was visible in central vacuoles of leaf epidermis, in conducting tissue and in root hairs, while GFPT was visible in central vacuoles of mesophyll cells and in stomates. In other tissues GFPT was visible in small compartments that could be either intermediary compartments or small vacuoles (8). These transgenic plants will be very useful for further studies of vacuolar targeting in plants.

References:

1. K. Matsuoka & J.-M. Neuhaus, *J Exp. Bot.* **50**, 165-174 (1999).

2. N. Paris *et al.*, *Plant Physiol.* **115**, 29-39 (1997).

3. N. Paris *et al.*,, *Cell* **85**, 563-572 (1996).

4. G. Y. Jauh, *et al.*, *Plant Cell* **11**, 1867-82 (1999).

5. G.-P. Di Sansebastiano, *et al.*, *Plant J.* **15**, 449-457 (1998).

6. G. P. Di Sansebastiano, PhD thesis, Neuchâtel (1999),

7 M. R. Schroeder, *et al.*, *Plant Physiol.* **101**, 451-458 (1993).

8 R. Flückiger, Diploma thesis, Neuchâtel (1999)

Poster T1

ABC TRANSPORTERS IN PLANTS: LINKING GENES AND FUNCTION

Markus Klein[$], Lucien Bovet[$], Nicola Gaedeke[$], and Enrico Martinoia[$]

[$]Université de Neuchâtel, Institut de Botanique, Lab. Physiologie végétale, Rue Emile Argand 13, CH-2007 Neuchâtel (e-mail: Markus.Klein@bota.unine.ch) and [§]Max-Planck-Institut für Molekulare Pflanzenphysiologie, Golm, Germany

According to the prevailing chemioosmotic theory, two vacuolar proton pumps generate an electrochemical potential across the tonoplast which can subsequently be used to energise the uphill transport of substances into the vacuolar lumen. The recent discovery of directly energised ATP-driven transport processes occurring on vacuolar membranes has suggested the presence of another class of pumps belonging to the family of ATP-binding cassette (ABC) transporters in plants. Preferential substrates for directly energised transport processes characterised in our group belong to the class of conjugated organic anions. Interestingly, some of the substrates like glutathione conjugates or certain glucosides are typical detoxification products of xenobiotic compounds. It has been suggested that ABC transporters are also involved in the vacuolar storage of secondary compounds. Recently, we have demonstrated that flavone glucuronides appearing specifically in the rye mesophyll and a non-plant glucuronide, ß-estradiol 17-(ß-D-glucuronide), are taken up into vacuoles from different plant sources by direct energization. Both substrates exhibit complex interaction patterns with glutathione and its conjugates showing as well competition as stimulation arguing for transport regulation by glutathione. However, it cannot be generalised that ABC transporters are responsible for flavonoid transport since different flavone glucosides from *barley* mesophyll are transported into *barley* vacuoles by an H^+-antiport mechanism while an ABC transporter is responsible for the transport of these substrates into *Arabidopsis* vacuoles, a plant not containing these compounds. Furthermore, the uptake of a herbicide glucoside used as a model substrate for abiotic glucosides into barley vacuoles also shows characteristics of ABC transporters. Presently, the molecular nature of the ABC transporter for glucosides is unknown.

In contrast, there is accumulating evidence that the subfamily of multidrug resistance associated proteins (MRP) which was originally identified in a drug resistant small cell lung cancer line is responsible for the ATP-dependent transport of conjugated organic anions e.g. during the elimination of catabolites in the liver into bile. Recently, the presence of homologs of human MRP's have been characterised in yeast and plants. The yeast cadmium factor 1 ycf1 functions as a glutathione conjugate pump and restores cadmium resistance by transporting $(glutathione)_2$-Cd-complexes. We are presently characterising another yeast MRP-like transporter which is responsible for the transport of glucuronides. Our group is currently cloning and expressing all known MRP-like genes identified in the *Arabidopsis* sequencing project and we investigate the expression and localisation in the plant. The expression of AtMRP3 isolated from *Arabidopsis* is strongly induced by herbicide treatment. This ABC protein transports glutathione conjugates and chlorophylle catabolites. AtMRP5, which is preferentially expressed in siliques, exhibits a transport activity with glucuronides after heterologous expression in yeast. For AtMRP3 and AtMRP4, cadmium resistance is restored in the Dycf1 mutant yeast. From RT-PCR data it seems obvious that AtMRP4 and AtMRP7 may play an important role in heavy metal detoxification.

In collaboration with U. Kolukisaoglu and B. Schulz (University of Cologne) we have isolated T-DNA tagged *Arabidopsis* plants in most of the known AtMRP's using a reverse genetic approach. Interestingly, the knockout mutant for AtMRP5 exhibits a clear reduction in root growth while the hypocotyl length is not affected. We will present first experiments to understand the physiological functions of MRPs by analysis of this and other knockout mutants and discuss new results concerning the inhibition of MRPs by sulfonylureas which are substances known to interact with ion channels and channel regulators.

References:

Klein, M., Weissenböck, G., Dufaud, A., Gaillard, C., Kreuz, K. and Martinoia, E. (1996) Different energization mechanisms drive the vacuolar uptake of a flavonoid glucoside and a herbicide glucoside. *J. Biol. Chem.* 271, 29666-29671

Klein, M., Martinoia, E. and Weissenböck, G. (1997) Transport of Lucifer Yellow CH into plant vacuoles - Evidence for direct energization of a sulphonated substance and implications for the design of new molecular probes. *FEBS Lett.* 420, 86-92

Klein, M., Martinoia, E. and Weissenböck, G. (1998) Directly energized uptake of ß-estradiol 17-(ß-D-glucoronide) in plant vacuoles is strongly stimulated by glutathione conjugates. *J. Biol. Chem.* 273, 262-270

Klein, M., Martinoia, E.; Hoffmann-Thoma, G. and Weissenböck, G. A membrane-potential dependent ABC-like transporter mediates the vacuolar uptake of rye flavone glucuronides - Regulation of glucuronide uptake by glutathione and its conjugates. *Plant J.* in press

Rea, P.A., Li, Z.-S., Lu, Y.-P., Drozdowicz, Y.M. and Martinoia, E. (1998). From vacuolar GS-X pumps to multispecific ABC transporters. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 727-760

Poster T2

EXPRESSION OF *LEMNA MINOR* APS REDUCTASE IN *A. THALIANA*

P. Spielhofer, S. Täschler, R. Op den Camp and C. Brunold

Institute of Plant Physiology, Altenbergrain 21, 3013 Berne

Sulfur assimilation is a critical pathway by which plants synthesize a wide variety of sulfur-containing organic compounds of central importance to growth and metabolism. Adenosine 5'-phosphosulfate reductase (APR) is considered to be the key enzyme of the assimilatory sulfate reduction (Brunold, 1993) because of its strategic position at the beginning of the pathway. cDNAs of this enzyme has been cloned from *A. thaliana* and *L. minor* (Suter et al. 2000).

The aim of this work is to determine the metabolic flux control coefficient of the APR. To achieve this goal, we created transgenic *A. thaliana* plants expressing APR from *L. minor* (Fig. 1). Interestingly, preliminary results indicate that these transgenic plants show increased amount of glutathione (Fig.2). Antisense technology will be used to generate transgenic plants with reduced APR activity. The obtained transgenic plants showing small but finite activity changes will allow us to determine the flux control coefficient directly from flux and activity measurement.

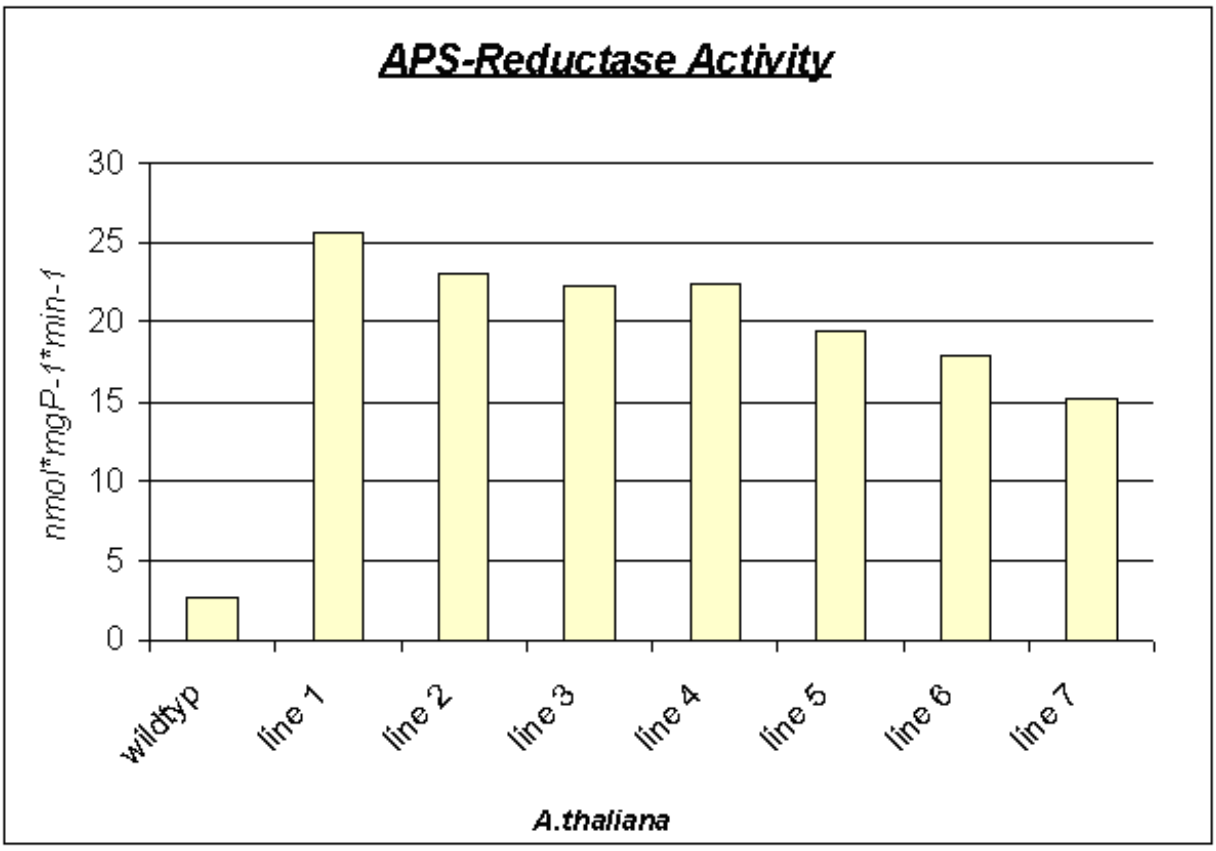


Fig.1: APR activity of transgenic *A. thaliana* plants expressing APS reductase from *L. minor*.

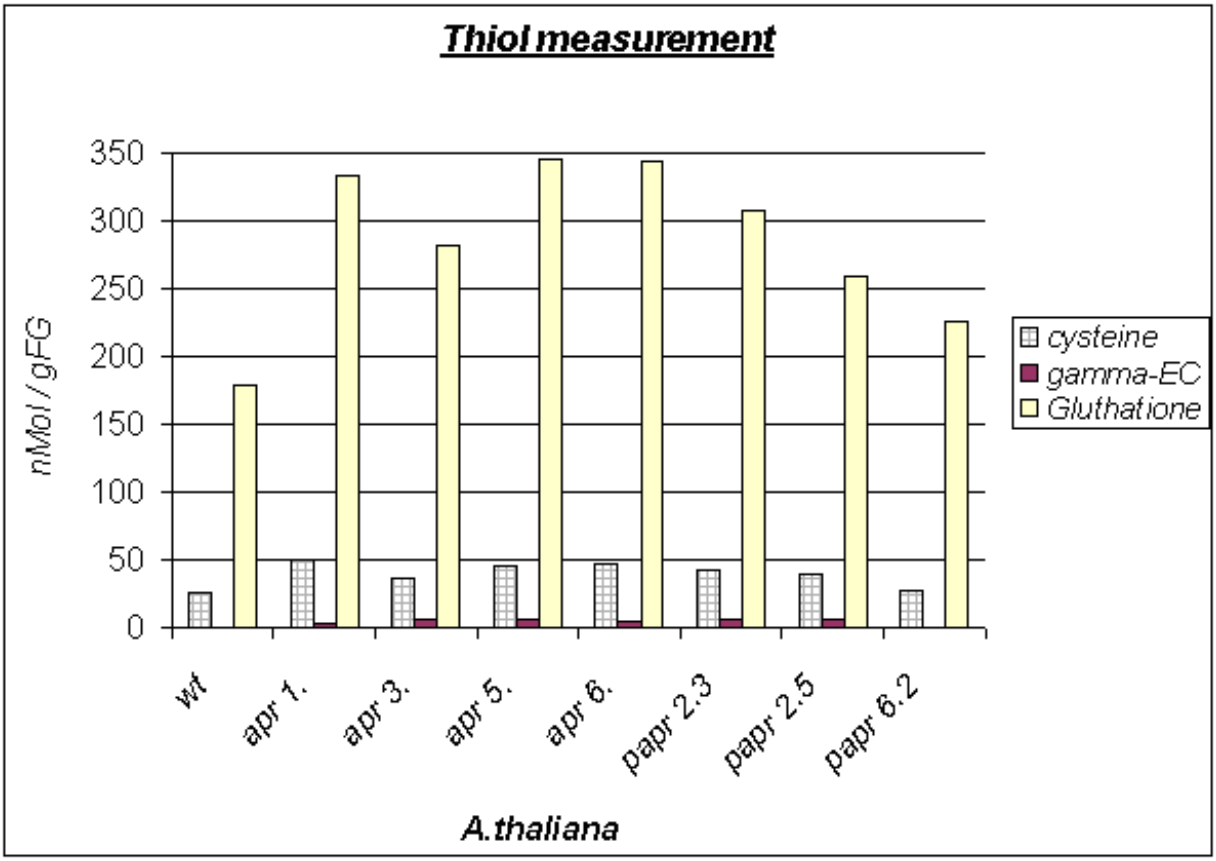


Fig.2: Thiol measurement of transgenic *A. thaliana* plants expressing APS reductase from *L. minor* and PAPS reductase from *E. coli*, respectively.

Literature:

C. Brunold: Regulatory interactions between sulfate and nitrate assimilation. In *Sulfur Nutrition and Sulfur Assimilation in Higher Plants* 1993 p. 61-75

M. Suter, P. von Ballmoos, S. Kopriva, R. Op den Camp, J. Schaller, C. Kuhlemeier, P. Schurmann, C. Brunold: Adenosine 5'-phosphosulfate sulfotransferase and adenosine 5'-phosphosulfate reductase are identical enzymes. J. Biol. Chem. 2000 Jan 14, 2000 275(02). 930.

Poster T3

REGULATION OF ASSIMILATORY SULFATE REDUCTION BY THIOLS IN ARABIDOPSIS THALIANA.

Pierre Vauclare, Mariane Suter, Sara Täscchler & Christian Brunold

Adenosine 5'-phosphosulfate reductase (APR) is considered to be the key enzyme of sulfate assimilation in higher plants by transferring the sulfonyl group of adenosine-5'-phosphosulfate (APS) produced by the ATP sulfurylase to an endogenous sulfite carrier. This enzyme constitutes the limiting step in the synthesis of cysteine and glutathione. For this reason, it ought to be considered as the principal site of the assimilatory sulfate regulation (Brunold, 1990; Rennenberg and Brunold, 1994, Brunold and Rennenberg, 1997). The effects of thiols on the regulation of sulfate assimilation were analysed in Arabidopsis thaliana roots cultivated in vitro. We demonstrated that an addition of L-cysteine in root nutrient solutions significantly reduced the APR activity, whereas no notable variation was observed on the ATP sulfurylase activity. However, in the presence of an inhibitor of ?-glutamyl-cysteine-synthetase (buthionine-S, R-sulfoximine : BSO) which prevents the formation of gluthatione, an increase of the extractable APR activity was observed. Under the same conditions, addition of reduced glutathione in the root medium resulted in an important decrease of the APR activity, whereas nothing was observed when the roots were simultaneously incubated with L-cysteine and BSO. This provided evidence that glutathione and not L-cysteine appears to be responsible for the loss of APR activity. Moreover, $^{35}SO_4^{2-}$ feeding experiments showed that in vivo incorporation of S^{35} into reduced thiols and proteins strongly decreases when the internal concentration of gluthatione was high. Indeed, an ~80 % reduction was noted for labelled proteins and thiols in incubated roots as compared with the control. Interestingly, a strong correlation was observed between the activity and the abundance of proteins and their corresponding transcripts. A decrease in inverse proportion to the internal concentration of gluthatione of all three APR isoforms mRNAs was evident.

Therefore, we conclude that the reduced glutathione pool (an end-product of the sulfate assimilation pathway) appears to serve as a negative signal responsible for sulfate assimilation by precisely regulating the APR isoforms gene expression at the transcriptional level.

References :

Brunold, C. (1990). Reduction of sulfate to sulfide. In : Rennenberg, H., Brunold, C., De Kok, L. J., and Stulen, I. (eds.), Sulfur nutrition and sulfur assimilation in higher plants, SPB Academic Publishing, The Hague, The Netherlands, pp 53-65.

Brunold, C. & Rennenberg, H. (1997). Regulation of sulfur metabolism in plants : first molecular approaches. Prog. Bot. 58, 164-186.

Rennenberg, H. and Brunold, C. (1994). Significance of glutathione metabolism in plants under stress. Prog. Bot. 55, 144-156.

Poster T4

A PLANT RECEPTOR FUNCTION DEMONSTRATED IN VIVO IN SACCHAROMYCES CEREVISIAE FOR TARGETING TO THE LYTIC VACUOLE.

Nadine Paris, David Humair, Doramys Hernandez and Jean-Marc Neuhaus.

Laboratoire de Biochimie, Emile-Argand 9, CH-2007 Neuchâtel, Switzerland.

Abstract

The plant secretory system is far less easy to study than its yeast counterpart mainly due to the lack of any equivalent plant mutant collection. Plant and yeast, in opposition to mammalian cells, do not use the mannose 6P-based lysosomal sorting tag but, instead, vacuolar sorting informations are carried by peptidic sequences. In yeast, the two vacuolar proteins carboxypeptidase Y (CPY) and proteinase A are both produced with a propeptide localised on their N-terminus and responsible for the vacuolar location of the mature protein. Almost fifty mutants, that are defective for vacuolar protein sorting (vps), allowed the identification of proteins and mechanisms for the yeast vacuolar sorting pathway. One of these proteins, Vps10p, plays a central role since it is the vacuolar sorting receptor for CPY (1). Vps10p is also able to send malfolded proteins to the vacuole for degradation in a process that is believed to be independant of any sorting signal. In plants, two main vacuolar sorting determinants (VSD) have been well studied and are believed to correspond to two separate vacuolar sorting pathways. The first group, with its mainly studied example barley aleurain (2), could be defined as a sequence specific type VSD with the conserved tetrapeptide NPIR that is also found in the other vacuolar protein sporamin A (3). The second group corresponds to short VSDs, with no obvious sequence conservation but need to be at the C-terminus of the soluble vacuolar protein. In this second category fall chitinase A (4) and barley lectin VSDs (5).

Very few is know about the pathway that uses VSDs with non conserved sequence beside its higher sensibility to wortmannine, a PI3-kinase inhibitor, in BY2 tobacco cell line (6). In opposition, a good candidate for a vacuolar targeting receptor implicated in a sequence specific pathway have been identified, BP-80 (binding protein of 80kDa). BP-80 has first been isolated from a pea clathrin coated vesicles (CCV) extract by its ability to bind *in vitro* the VSD from aleurain. This binding is pH sensitive since it occurs at neutral pH and is abolished at pH4. Binding assay also showed the specificity of interaction between BP-80 and its ligand since the VSD from sporamin A was partially able to compete with aleurain while barley lectin VSD is unable to such an effect. BP-80 is a type I protein with the N-terminal domain involved in recognising aleurain sorting determinant (7). BP-80 was then further characterised and cloned (8). In addition to the CCV, it is localised in region emerging from the Golgi apparatus as well as in small structures next to the central vacuole, assumed to be prevacuoles. Along with its cloning, several homologues were identified in different species including arabidopsis, rice and maize. BP-80 was therefore renamed vacuolar sorting receptor from *Pisum sativum*-1, VSR-PS1, in order to distinguish this protein from its variants (8). Although all the BP-80 characteristics are in favour of its role as a vacuolar receptor the function itself is not yet proved.

We set up a test *in vivo* in yeast in order to demonstrate the vacuolar targeting function of VSR-PS1. We used a strain deficient for Vps10p, the yeast vacuolar sorting receptor. By coexpressing the vacuolar sorting determinant of aleurain fused to the reporter protein GFP (green fluorescent protein) together with the plant receptor, we were able to show specific accumulation of the GFP inside the yeast vacuole. We not only demonstrated that VSR-PS1 does interact with its ligand *in vivo* but also that this interaction leads to the transport of our reporter to the yeast vacuole. In addition, this test can be used to adress the specificity of interaction between VSR-PS1 and its ligand *in vivo*. These results also underlight high similarities between yeast and plant secretory systems since a plant receptor by exposing its own trafficking signals is properly handled by yeast secretory pathway machinery. We are now in the process of studying the range of vacuolar sorting determinants recognised by VSR-PS1 as well as by some of the numerous homologues cloned so far.

References:

1. Marcusson, E. G., Horazdovsky, B. F., Cereghino, J. L., Gharakhanian, E. & Emr, S. D. (1994) Cell 77, 579-786.

2. Holwerda, B. C., Padgett, H. S. & Rogers, J. C. (1992) Plant Cell 4, 307-318.

3. Matsuoka, K. & Nakamura, K. (1991) Proc. Natl. Acad. Sci. USA 88, 834-838.

4. Neuhaus, J.-M., Sticher, L., Meins, F. & Boller, T. (1991) Proc. Natl. Acad. Sci. USA 88, 10362-10366.

5. Bednarek, S. & Raikhel, N. V. (1991) Plant Cell 3, 1195-1206.

6. Matsuoka, K., Bassham, D. C., Raikhel, N. & Nakamura, K. (1995) J. Cell. Biol. in press,

7. Kirsch, T., Paris, N., Butler, J. M., Beevers, L. & Rogers, J. C. (1994) Proc Natl. Acad. Sci. USA 91, 3403-3407.

8. Paris, N., Rogers, S. W., Jiang, L., Kirsch, T., Beevers, L., Phillips, T. E. & Rogers, J. C. (1997) Plant Physiology 115, 29-39.