# Structure, Physiology and Molecular Biology

# MODULATION OF SA-PERCEPTION BY PHYTOCHROME SIGNALING IN *ARABIDOPSIS*

*Thierry Genoud and Jean-Pierre Métraux*

Département de Biologie, Université de Fribourg, Switzerland

Interference of light perception in the defense signal transduction pathway has been observed in *Arabidopsis* (Genoud et al., 1998; Genoud and Métraux, 1999). We used mutants affected in light perception and light signal transduction to investigate this phenomenon. The response of plants to SA and functional analogs appears to be strictly correlated to the phytochrome signal but not to the carbohydrate content (Fig.1).

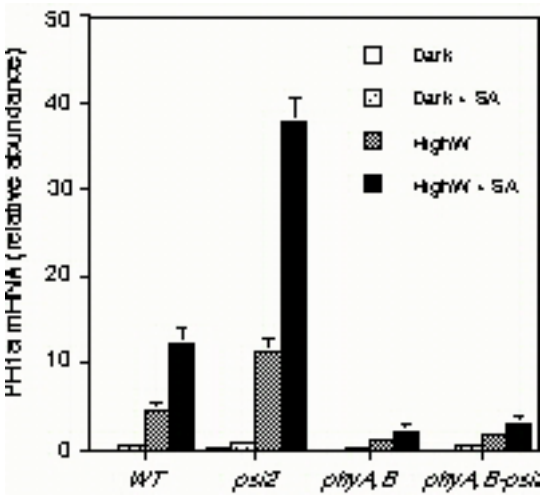



Fig.1 PR1a expression in WT, *psi2*, *phyA-phyB*, and triple mutant *phyA-phyB-psi2* grown in darkness or under high white light fluence rate (HighW; 100 mmol $m^{-2}s^{-1}$) on a sucrose-containing medium. Plants were treated 5 days after germination with 10 mM SA. Total RNA was extracted 48 h after the treatment and used for gel blot analysis.

Moreover, SA has no activity on PR gene induction in absence of light, and generates very low effects in dim light (Fig.2).

The initiation of rapid cell death, hallmark of the plant hypersensitive response to pathogens (HR), also exhibits a strict dependence on both the presence and amplitude of a phytochrome-elicited signal. Our results demonstrate that the phytochrome signal crosstalks with SA signaling and potentiates a light-dependent process of defense.

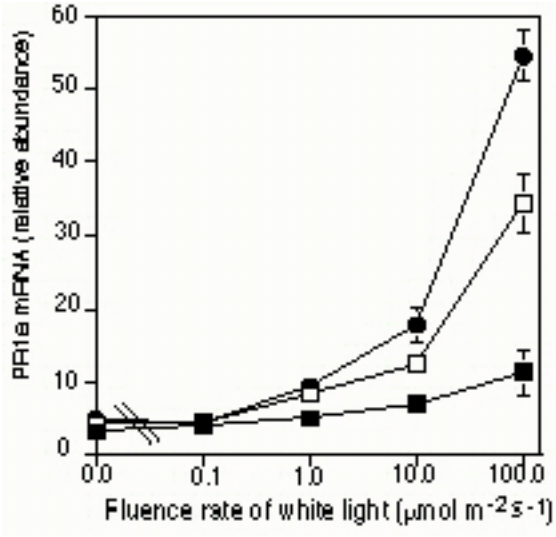



Fig.2 PR1a expression in 7-day-old seedlings grown on sucrose-containing medium.WT seedlings grown under various fluence rates of white light were treated with SA (10 mM; open squares) or BTH (1 mM; closed circles), closed squares: control. Total RNA was extracted 48 h after treatment and used for gel blot analysis.

A new genetic screen has been elaborated in order to isolate mutants presenting a defective modulation of the SA-controlled pathway by the phytochrome-generated signal. Partial and complete reversion of the spontaneous light-depending HR-like necroses under high irradiation of white light have been found in mutants resulting from a EMS treatment of *psi2* seeds. Plants failing to develop spontaneous necroses under screening conditions were isolated and their responses to light and SA were scored. Recessive and dominant mutants with a reduced transduction of particular light and defense signals have been characterized. This group of genetic lesions called *ltd* (light-to-defense) defines important elements of the cell signaling apparatus involved in the light control of PR-gene expression and HR development.

Genoud, T., Millar, A.J., Nishizawa, N., Kay, S.A., Schäfer E., Nagatani, A., and Chua, N.-H. **(1998). Plant Cell** 10**, 889-904.**

Genoud, T., and Métraux, J.-P. **(1999). Trends Plant Sci.** 4**, 503-507.**

# ARE GLUTATHIONE-TRANSFERASES BORING UNSPECIFIC ENZYMES?

*Ulrich Wagner, Damien Lieberherr, Jean-Pierre Métraux and Felix Mauch*

University of Fribourg, Department of Biology, 3 Rte Albert Gockel, CH-1700 Fribourg (felix.mauch@unifr.ch)

Glutathione-transferases (GSTs) are a ubiquitous enzymes that catalyze the addition of the tripeptide glutathione to a diverse range of hydrophobic compounds. GSTs have been studied intensively in animals and plants with regard to their detoxification potential towards xenobiotics. Little is known about their roles in normal plant metabolism. It is generally assumed that GSTs have a function in cellular protection in stress situations and that they play a role in the targeting process of secondary metabolites to the vacuole. The long term goal of our project is to learn more about the enigmatic functions of plant glutathione transferases using Arabidopsis as a model plant. The Arabidopsis GST gene familiy has turned out to be surprisingly complex. It currently includes 38 members and the final number is predicted to be close to 50! The GST family can be grouped into 4 rather different structural subclasses. The two larger subclasses appear to be plant specific while the two smaller subgroups are related to animal GSTs. Apart from sequence information, very little is known about GST gene regulation and enzyme activity let alone physiological function. Why are there so many GSTs? The GST family shows extreme sequence divergence with less than 20% amino acid identity between the most divergent members. Combined with the wide substrate range, this suggests that individual GSTs might have specific roles in protection from diverse stresses as well as roles in secondary metabolism, hormon homeostasis and plant development.

A set of twelve representative GSTs was chosen for analysis of gene regulation and enzymatic activity. Our results show that individual GST genes are differentially regulated in response to many biologically relevant stress conditions such as light conditions, phytohormone treatments, heavy metals, herbizides, ozone, wounding and pathogen attack. A subset of at least three GSTs is stronly induced following inoculation with *Pseudomonas syringae* (*P.s.*) or by the application of salicylate (SA). In accordance with an endogenous role of SA in the induction process, induction of GSTs by *P.s.* does not occur in *nahG* plants that are unable to accumulate salicylate. The induction of GST and PR genes are both SA-dependent. However, the induction of GSTs by *P.s.* is much faster (1-2h p.i.) than the induction of PR-1 gene expression (8h p.i.). Surprisingly, it appears as if in the early phase of infection the induction of enzymes of cellular protection is more urgent than the activation of defense genes. In the case of GST2, this increased sensitivity is achieved by the effect of ethylene. *P.s.* does not induce GST2 in the ethylene mutant *etr1*. Thus, full induction of GST2 is dependent on the synergistic activity of SA and ethylene. This example demonstrates that the mechanism of regulation of GST expression can be rather sophisticated. The mechsanism is also specific since the expression of no other GST is regulated in the same way.

Twelve GST cDNAs were functionally expressed in *E. coli* and tested for enzymatic activity with 6 diagnostic substrates. The deduced substrate profiles of individual GSTs were dramatically different indicating that the wide spread notion of GSTs being rather boring unspecific enzymes is not correct. The apparent broad substrate range of GSTs is not a property of the individual enzymes but a reflection of the diversity of GSTs present in plants. It seems as if this diversity is needed to counteract the many consequences of unfavourable conditions. Our results show that plant GSTs have specific preferences for the second substrate and can not be considered as a simple collection

of isozymes. As a consequence plant GSTs very probably have well defined metabolic functions. As an example: Many pathogen-induced GSTs have high glutathione peroxidase activity towards fatty acid hydroperoxides and are very likely involved in protection against the toxic consequences of oxidative stress. We postulate that these GSTs detoxify cytotoxic fatty acid hydroperoxides generated at infection sites and thus contribute to the local restriction of the toxic consequences of oxidative stress. This hypothesis is in line with emerging evidence that some GSTs have an inhibitory effect on apoptosis in other organisms. Our hypothesis will be tested by manipulating GST expression in transgenic plants.

# A LOAD OF PEROXIDASE GENES IN *ARABIDOPSIS THALIANA*

*Michaël Tognolli, Serge Overney, Claude Penel, Hubert Greppin and Patrice Simon*

Laboratoire de Biochimie et Physiologie Végétales, Université de Genève, 3 place de l'Université, 1211 Genève 4, email: patrice.simon@bota.unige.ch

Plants are known to possess numerous forms of the classical guaiacol peroxidase, referred to now as class III secretory peroxidase [1,2]. These forms can be pseudo-isozymes, generated by posttranscriptional and posttranslational modifications of a gene product, or true isozymes arising from gene duplication and diversification. Evidence for the multiplicity of peroxidase genes was observed in horseradish [3] and was shown in spinach by cDNA cloning with a conserved probe [4]. During the last few years, the search for peroxidase genes in the model plant *Arabidopsis thaliana* has lead to the identification of 70 genes for the moment and about 15-20 more are expected. Some peroxidases have been cloned in a few laboratories, but the majority of them have been harvested in the ongoing genome analysis projects, the Arabidopsis Expressed Sequence Tag sequencing projects and the Arabidopsis Genome Initiative.

*Genetic structure*. Phylogenic analysis shows a significantly high degree of evolutionary divergence and individualization within this multigene family, ranging from a few to more than 60 substitutions per 100 residues. Several genes appear as isolates or pairs, while a few clusters are easily distinguishable. The intron-exon structures show variations that support the concept of an ancestral gene containing 3 introns. Diversification arose during evolution by gene duplication assorted with loss and gain of introns. Four genes, closely related on the phylogenetic tree, are devoid of any introns, and have most likely evolved from an mRNA intermediate. Evidence for a mechanism of intron loss by replacement of an ancestral intron-containing gene with a reverse-transcribed DNA copy of a fully spliced mRNA has recently been discussed for catalase and alcohol dehydrogenase [5,6]. The varied intron patterns in peroxidase genes offer also a unique opportunity to challenge the role given to introns in the enhancement of transcription and stabilisation of mRNAs. Positioning of the genes on the chromosomes reveals a significant level of promiscuous intra- and inter-chromosomal gene shuffling. Genes with a high level of homology can be located very close to each other but not necessarily. However, genes with high linkage on the chromosome do not necessarily show high homology.

*Gene expression.* The transcript level of 23 peroxidases, all Ests, was assayed by *Northern* analysis in roots, stems, leaves and flowers of 14 and 28 day-old plants. A positive signal was obtained for 13 peroxidases in at least one of the tissues tested. The most usual and highest expression was found in roots, except for one peroxidase which was only detectable at a low level in stems and another one which was only seen in leaves and flowers. Two peroxidases were expressed unspecifically in all tissues, seven peroxidases in roots only, one in roots and flowers and one in roots and stem. Ten of them have no apparent expression in the investigated organs.

*Enzyme activity.* The identification of peroxidase genes relies on the homology of the encoded proteins with known peroxidase structures. The peroxidasic activity of the encoded proteins remains however to be demonstrated, and specific properties that could support potential roles and functions of the various enzymes *in planta* need to be established. In order to link the presumed peroxidase genes to their products, our approach is to express the genes in an appropriate heterologous system and to analyze the recombinant protein for specific properties and enzymatic capacities. For this purpose, we are expressing the *Arabidopsis* cDNAs in the baculovirus/insect cells system, which has already been used efficiently to express horseradish peroxidase [7]. Twenty-one

peroxidases were expressed as recombinant proteins in the baculovirus/insect cells heterologous system. Fourteen of them yielded active enzymes, which were detected by measuring their activity in the spent medium. Oxidation of the substrates *o*-phenylenediamine, ABTS and guaiacol showed significant specificities, which can be strongly modulated by pH and calcium ions. For the 7 remainders uncomplete/erroneous posttraductional processing or peculiar substrate specificity are possible reasons for undetectable activity with the standard peroxidase substrates. We have also collected preliminary evidences that certain isoperoxidases possess specific activities with substances considered as being possible natural substrates like ferulic acid, scopoletin and extensin and for the capacity to produce hydrogen peroxide with a reducing substrate like cysteine [8]. The specific binding to natural substrate has also been observed.

*Conclusions.* Despite the small size of its genome, *Arabidopsis thaliana* carries an important load of isoperoxidases that evolution has multiplied and sprinkled over its 5 chromosomes. *Northern* analysis has shown that most of the clones are expressed in the developmental conditions investigated and display specific patterns. Roots appear as a privileged site to encounter numerous peroxidases. Fourteen active peroxidases were produced in the baculovirus/insect cell system The major drawback of our expression system is the difficulty encountered in purifying significant amount of the recombinant proteins. This problem can be easily circumvented by putting a tag to the protein and using it as a purification bait. Our observations suggest that the peroxidase multigene family has apparently evolved by gene duplication, diversification and acquisition of new enzymatic capacities and protein properties. The specificities observed at the various levels, namely RNA expression, substrate consumption, hydrogen peroxide generating capacity, calcium control of activity and binding to natural polymers, favor the concept that isoperoxidases have a specific role to play under appropriate conditions at the right time and at the right place.

*Acknowlegments.* We are thankful to the ABRC at the Ohio StateUniversity and to Dr T. Newman at the MSU for providing the Est clones. This work is part of a joint EU-project (AIR2-CT93-1661) and was supported by the Swiss Federal Office of Education (grant OFES 93.0090).

References:

**1**. Gaspar Th, Penel C, Thorpe T, and Greppin H (1982) *Peroxidases 1970-1980,* University of Geneva, Switzerland.

**2**. Welinder KG (1992) Curr Opin Struct Biol 2: 388-393.

**3**. Welinder KG (1992) in *Plant Peroxidases 1980-1990, Topics and Detailed Literature on Molecular, Biochemical, and Physiological Aspects* (Penel C, Gaspar Th, Greppin H. eds) University of Geneva, pp 1-24.

**4**. Simon P (1993) *Plant Peroxidase Newsletter* (Penel C, Gaspar Th, Greppin H, eds), 1**,** pp 4-7, University of Geneva, Switzerland.

**5**. Frugoli JA, McPeek MA, Thomas TL, and McClung CR (1998) *Genetics* 149: 355-365.

**6**. Charlesworth D, Liu FL, and Zhang L (1998) *Mol. Biol. Evol.* 15: 552-559.

**7**. Hartmann C, and Ortiz de Montellano PR (1992) *Arch. Biochem. Biophys.* 297: 61-72.

**8**. Overney S, Tognolli M, Simon P, Greppin H, and Penel C (1998) *Bull. Soc. Roy. Sc. LiËge* 67: 89-98.

# SEARCHING FOR NOVEL BIOLOGICAL ACTIVITIES OF OXYLIPINS IN PLANTS.

*Edward E. Farmer, Hans Weber, Sabine Vollenweider, Philippe Reymond, Emmanuelle Alméras and Aurore Chételat.* Institute of Ecology, Plant Biology and Physiology, Biology Building, CH-1015 Lausanne, Switzerland.

Oxylipins (oxygenated fatty acid derivatives) play diverse roles in plant biology as signal molecules for defense gene expression and as antimicrobial compounds. Many oxylipins are generated by the action of lipoxygenases. In plants, these enzymes add molecular oxygen to pentadiene fatty acids, such as linoleic acid and linolenic acid. The resultant products, fatty acid hydroperoxides, are subject to a diverse array of modifications leading to the generation of large numbers of other oxylipins whose function have not been definitively determined. A family of potent biological regulators in plants, the jasmonate family, is, however, known. Jasmonates regulate the expression of a number of genes necessary for the defense of plants against insect and microbial pathogens. In order to search for novel, biologically active oxylipins in plants our laboratory developed the 'oxylipin signature' technique (Weber et al., PNAS 94, 10473; Weber et al., Plant Cell 11, 485). This method allows a global analysis of the oxylipin pool. The oxylipin signature method simply involves extraction of tissues and partial purification of the oxylipin fraction, which is enriched in oxidized fatty acids containing between 12 and 18 carbons. These compounds are separated by gas chromatography and analyzed by mass spectrometry. With the aid of the method, a new 16-carbon member of the jasmonic acid family, known as dinor-oxo-phytodienoic acid (dnOPDA), was recently discovered in potato and *Arabidopsis* tissues (Weber et al. PNAS, 94, 10473).

In a different study, we applied the oxylipin signature method to late blight of potato, one of the world's most problematic plant diseases. The causal agent, *Phytophthora infestans*, a fungal protist, was a principal cause of the devastating potato famine in Ireland in the mid-1800s. We studied dynamic changes in oxylipin profiles of late-blight diseased potato leaves, concentrating particularly on 9-lipoxygenase-derived oxylipins. This study revealed the presence of divinyl ether fatty acids for the first time in higher plants and these compounds were found to be inhibitory to the late blight pathogen (Weber et al., Plant Cell 11, 485).

Our laboratory is now extending the search for novel biologically active oxylipins concentrating on a search for novel regulators of gene expression. Lipid peroxidation in many organisms results in the generation of widely occurring and characteristic products including fatty acid ketenes. Two of these compounds were observed to accumulate in *Arabidopsis* leaves infected with *Pseudomonas syringae*. Little is known about the regulatory effects of these ubiquitous compounds. We investigated the potential regulatory activity of fatty acid ketenes in *Arabidopsis*. In this plant, several genes including a glutathione *S*-transferase encoding gene, *GST1*, are known to be powerfully activated in pathogenesis under conditions where lipid peroxidation takes place. Ketenes were found to activate *GST1* and are thus candidates for a new class of biologically active oxylipins (submitted).

We have now incorporated cDNA microarrays into our program to find novel biologically active oxylipins in plants. *Arabidopsis* mutant analyses, data from oxylipin signature experiments, and cDNA microarray analysis are being combined in this effort to dissect the complexity of signal transduction pathways in *Arabidopsis* (Reymond et al., submitted) and attribute functions to some of the hundreds of oxylipins known in plants and other eucaryotes.

# Poster S1

## IDENTIFICATION OF A PECTIN BINDING SITE ON AN EXTRACELLULAR PEROXIDASE

*C. Penel, S. Carpin, M. de Meyer, P. Simon and H. Greppin*

Laboratoire de Physiologie végétale, Université de Genève, 3 place de l'Université, 1211 Genève 4, Switzerland, email: Claude.Penel@bota.unige.ch

A same plant may contain up to nearly a hundred of genes encoding a classical secretory peroxidase. Each of these genes seems to be regulated differently. Expression studies have shown that the transcription is often tissue-specific and developmentally regulated [1,2] but it can also be dependent on various external stimuli [3]. Many isoperoxidase molecules are secreted and exert their function in cell walls, but very little is known concerning the control of their distribution within the extracellular matrix. Once they are released at the cell surface by exocytosis, they face the complex network formed by the cell wall polymers (Fig. 1).

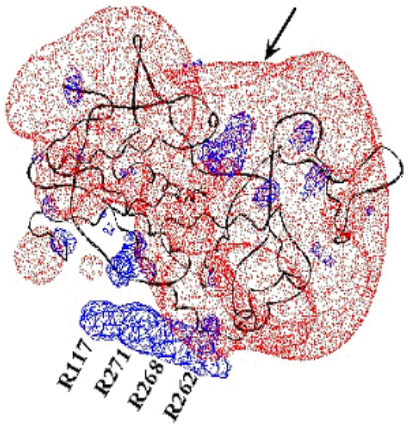



**Figure 1.** Electrostatic potential map of APRX. A homology model was prepared for APRX with the programs Swiss-Model and Swiss-Pdb viewer, using the structure of peanut peroxidase as a template. The protein is shown as a coil, the negative potentials by dots and positive potentials by lines. The arrow shows the substrate channel access.

: determination of their final localization, which can be :ial for their function, will therefore be mainly dependent :he affinity they may have for one of the cell wall stituents. Enzyme molecules without any affinity will probably diffuse randomly throughout the extracellular matrix. As peroxidases are important for the control of cell wall structure, through the cross-linking of many cell wall polymers or through lignin and suberin deposition, a control of the spatial distribution of these enzymes seems however to be necessary.

Among the very few cases of extracellular proteins reported to be able to interact specifically with a cell wall polymer are some isoperoxidases from zucchini, horseradish or Arabidopsis [4,5]. These proteins have shown a strong affinity for the homogalacturonan chains of pectin cross-linked by calcium ions (Ca-pectate)[4]. This property could be important for focusing these enzymes within some precise cell wall domains rich in unesterified pectin such as the middle lamella, and also in some tissue such as the epidermis [5]. An anionic isoperoxidase from zucchini (APRX) exhibiting this particular binding property has been studied with some details. It turned out that its strong affinity for Ca-pectate was due to the presence of cationic amino acid residues [4]. APRX was purified, microsequenced and cloned. *In situ* hybridization experiments revealed that the *APRX* gene is expressed mainly in dermal tissues, in some cells of the vascular system or in the cotyledon palisade parenchyma [1]. The deduced amino acid sequence of APRX was used to build up a homology 3-D model based on the known crystal structure of peanut peroxidase [6]. The model shown in Figure 1 displays the electrostatic potential map at the surface of the protein. It clearly appears that the surface of APRX is mostly negative except a small positive area formed by the alignment of four arginine residues. This cationic domain located in the peroxidase molecule at the opposite of the substrate channel entry is likely to be responsible for the affinity to Ca-pectate.

This hypothesis was tested by site-directed mutagenesis, using recombinant peroxidases synthesized by the insect cells/baculovirus expression vector system. The molecular weight and isoelectric point

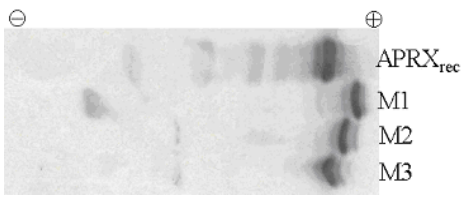



**Figure 2.** Isoelectric focusing separation of recombinant zucchini peroxidases (55 ng) obtained from transfected insect cells. M1-M3: APRX mutants. The gel was stained for peroxidase activity with *o*-dianisidine-$H_2O_2$.

of the recombinant peroxidase expressed in insect cells (APRX$_{rec}$) were similar to those of the plant APRX. In addition, the recombinant protein binds to Ca-pectate with an affinity equal to the affinity exhibited by the plant enzyme. Mutated *APRX* sequences were produced using the GeneEditor *in vitro* site-directed mutagenesis system from Promega Three mutations were designed to convert up to three arginines in serine or glutamine by a single base substitution for amino acid: M1: R117S; M2: (R262S/R268S); M3: (R262S/R268S/R271Q). The mutated APRX expressed in insect cells had a catalytic activity and a molecular weight similar to those of APRX or APRX$_{rec}$ As can be expected, their isoelectric point was somewhat affected by the removal of one, two or three arginines, showing a corresponding shift towards more acidic pH (Fig. 2). The three APRX mutants were then assayed for their ability to bind Ca-pectate (Table 1). The results showed that the mutation of the three arginine residues (M3) located on the same a-helix (J) abolished the affinity for Ca-pectate. The substitution of two arginines (M2) lowered the affinity, whereas the withdrawal of only one arginine (M1) had no effect. This observation seems to indicate that the three arginines on J helix are necessary and sufficient for APRX to bind to pectin in the presence of calcium ions, forming thus a binding site. This site could be used to target proteins towards cell wall domains rich in pectins. Further work is in progress to understand the function of this binding in cell wall.

1: Carpin S, Crèvecoeur M, Greppin H, Penel C (1999) Plant Physiol 120:799-810.

**Table 1.** Percentage of peroxidase binding to polygalacturonic acid (10 µg) in the presence of 2 mM $CaCl_2$

| Peroxidase | per cent |
|---|---|
| APRX$_{rec}$ | 99.5 |
| M1 | 99.2 |
| M2 | 62.1 |
| M3 | 3.7 |

**2:** Klotz JP, Liu TT-Y, Liu L, Lagrimini M (1998) Plant Mol Biol 36:509-520.

**3:** Kawamoto T, Kawaoka A, Yoshida K, Sekine M, Takano M, Shinmyo A (1993) Plant Cell Physiol 34

(Suppl): s32;

**4:** Penel C, Greppin H (1996) Plant Physiol Biochem 34:479-488.

**5:** Penel C, Crèvecoeur M, Greppin H (1996) *in* Plant Peroxidases. Biochemistry and Physiology

(Obinger C, Ebermann R, Penel C and Greppin H, eds) pp. 259-263, University of Geneva,

Switzerland;

**6.** Schuller DJ, Ban N, Van Huystee RB, McPherson A, Poulos TL (1996) Structure 4:311-321

# Poster S2

## GENETIC APPROACH TOWARDS THE ELUCIDATION OF THE POSSIBLE ROLE OF REACTIVE OXYGEN AS A SIGNAL IN STRESS RESPONSES.

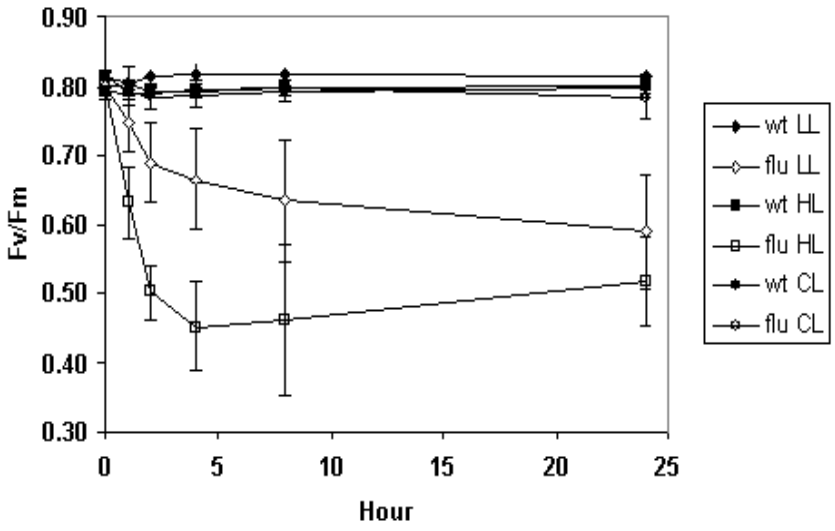
*R. op den Camp, C. Ochsenbein, D. Loew, M. Nater, K. Apel*

Institute of Plant Sciences, ETH Zürich

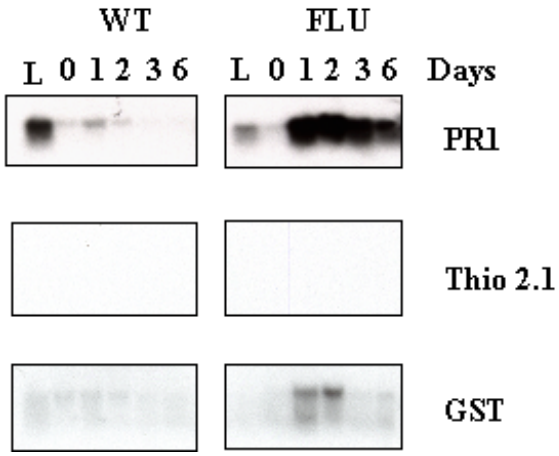
Photosynthetic organisms are prone to oxidative stress. Upon illumination excited porphyrin molecules such as chlorophyll may transfer the excitation energy directly to oxygen, thus leading to the formation of highly reactive singlet oxygen. Most of the chlorophyll is bound to proteins and in this state may use various quenching mechanisms to dissipate absorbed light energy. Its biosynthetic precusors, however, occur mostly in a free form and are potentially much more destructive when illuminated. In etiolated seedlings of angiosperms tetrapyrrole biosynthesis is blocked once a critical level of protochlorophyllide, the immediate precursor of chlorophyll, has been reached to prevent oxidative damage.

We have isolated the *flu* mutant that has lost the ability to suppress protochlorophyllide accumulation in the dark. This mutant is extremely sensitive to photooxidative stress and rapidly shows lesions once it is exposed to light. It was shown that the formation of the lesions is depending on the accumulation of protochlorophyllide (by putting the mutant in the dark) and the light intensity by measuring the Fv/Fm (which is an indication for the efficiency of the photosystem II) (Figure 1).

Figure 1: The effect of low and high light on the *flu* mutant. CL= continous light, HL=high light and LL=low light.



The accumulation of protochlorophyllide in the dark and the subsequent exposure to light will lead to the production of reactive oxygen species. These reactive oxygen species are responsible for the induced cell death in the *flu* mutant. After induction of the lesion formation in the *flu* mutant we could show the induced expression of PR1 (pathogen induced gene) and GST1 (a gene induced by oxidative stress), but not of Thionin 2.1 (a wound induced gene) (Figure 2). Other typical stress reactions are the systemic induction of PR1 and the deposition of callose around the lesions. All these features are typical for a hypersensitive response normally induced by pathogens. The activation of the hypersensitive response indicates that the lesion formation in the *flu* mutant is not only caused by a direct toxic effect of reactive oxygen species but that intrinsic pathways for stress




responses are also activated.

Figure 2: Genes induced in the flu mutant after a dark-light treatment.

L= continous light, 0= transition from dark to light.

There are multiple biotic and abiotic stressors, such as cold, high light, UV light, heat, wounding and pathogens, which lead to specific stress responses. Most of these stressors seem to use reactive oxygen species as a component in the signal transduction between stress perception and final response. The way plants use reactive oxygen species for signal transduction is not known. We have carried out a second-site-mutant screen with the *flu* mutant. In this screen we have selected for suppressors of the *flu* phenotype, which still accumulate protochlorophyllide during a dark period (but are no longer showing the typical hypersensitive response). Through these revertants we hope to define genetically and biochemically the signal transduction pathway(s) through which reactive oxygen species may control the growth and development of higher plants.

# Poster S3

## IDENTIFICATION, CLONING, AND PROPERTIES OF CYTOSOLIC D-RIBULOSE-5-PHOSPHATE 3-EPIMERASE FROM HIGHER PLANTS

*Stanislav Kopriva, Anna Koprivova, and Karl-Heinz Süss§*

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

The oxidative pentose phosphate pathway (OPPP) is a major source of NADPH and substrates for the biosynthesis of various cell components such as nucleic acids, flavonoids, and proteins. Since OPPP enzymes in animal and yeast cells are located exclusively in the cytosol, some text books proposed an analogous location of this pathway in plant cells [1]. Reportedly, however, several essential OPPP enzymes could not be detected in the cytosol of plant leaf cells [2,3]. On the other hand, chloroplasts and chromoplasts contain the components for a complete OPPP [4,5]. Cloning of the chloroplast RPEase cDNA from potato [6] and spinach [7] thus opened up a way to look for other, possibly non-plastidic, isoforms of this enzyme.

The search of GenBank database with the potato chloroplast (chl) RPEase [6] revealed two groups of EST entries from rice (*Oriza sativa*) with 65% and 45% identity to the querry sequence, respectively. The former group most probably codes for a plastidic isoform of RPEase. The other cDNA clones coded for a protein of 24.3 kDa which was 40% identical in its primary structure with the rice and potato chl-RPEase, but possessed 52% identical residues with the cytosolic (cyt) RPEases from yeast and human. Notably, human cyt-RPEase and rice chl-RPEase share only 36% identical residues. The novel RPEase isoform from rice lacked a chloroplast targeting sequence indicating its localization in the cytosol.

The phylogenetic analysis, performed with the TREECON software [8], clearly revealed that plant chl-RPEase are closely related to the cyanobacterial homologue from *Synechocystis* (Figure 1) and, therefore, the nuclear gene encoding chl-RPEase was most likely acquired from the chloroplast ancestor [9]. The rice cyt-RPEase, however, is more closely related to the cytosolic enzyme homologues in yeast and animal cells than it is to any chl-RPEase. This finding thus suggests that cyt-RPEase genes originate from eubacteria and have not been replaced by cyanobacterial homologues in the course of plant cell evolution.

The expression analysis showed that cyt-RPEase transcript is predominantly accumulated in roots and seedlings, whereas only a small amount of mRNA was detectable in leaves (Figure 2). In contrast, chl-RPEase transcript was mainly expressed in leaves, and present only in minute quantities in seedlings and roots. Western blotting of proteins prepared from the same rice tissues corroborated the results of RNA analysis for cyt-RPEase, showing the presence of this enzyme in all plant organs with maximum accumulation in root cells. Similar to the results of Teige et al. [6], chl-RPEase protein could not be detected in root tissue at all, although a low level of transcript was observed there.

The first cloning of a cytoplasmic isoform of enzyme involved in the regenerative part of OPPP, therefore, support the view that a complete OPPP operates both in plastids and the cytosol of plant cells.

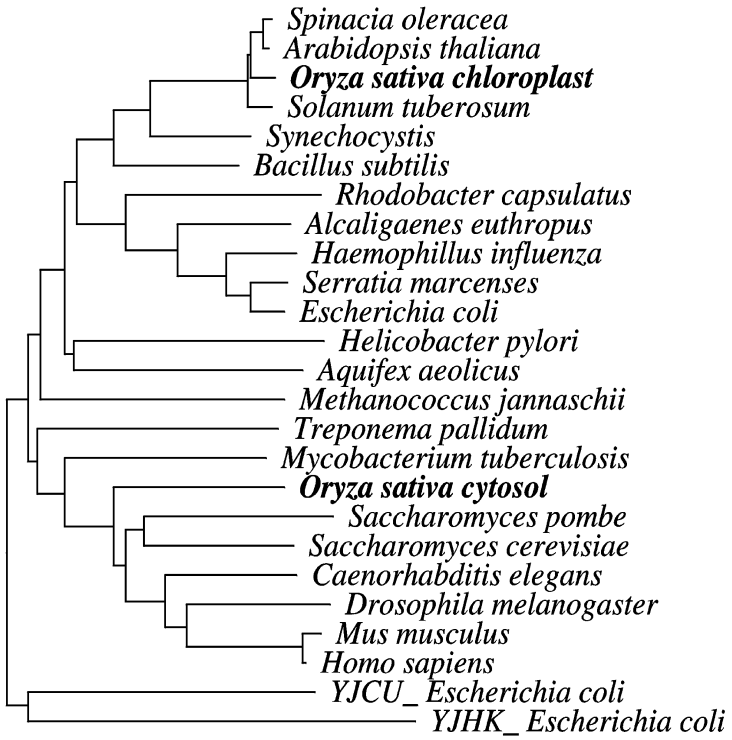



Figure 1. The neighbor-joining tree of RPEase amino acid sequences.

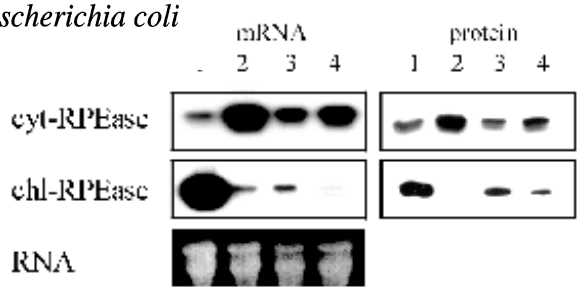



Figure 2. Expression analysis of RPEase in riceleaves (1), roots (2), and seedlings germinated in light (3) and dark (4).

References

Lüttge, U., Kluge, M., and Bauer, G. (1994) *Botanik*. WCH Verlagsgesellschaft, Weinheim

Feierabend, J., and Gringel, G. (1983) *Z. Pflanzenphysiol*. **110,** 247-258

Schnarrenberger, C., Flechner, A., and Martin, W. (1995) *Plant Physiol.* **108,** 609-614

Dennis, D.T., and Turpin, D.H. (1990) Plant Physiology, Biochemistry and Molecular Biology. Longman Scientific and Technical, Singapore

Thom, E., Mühlmann, T., Quick, P. W., Camara, B., and Neuhaus, H.-E. (1998) *Planta* **204,** 226-233

Teige, M., Kopriva, S., Bauwe, H., and Süss, K.-H. (1995) *FEBS Lett.* **377,** 349-352

Nowitzki, U., Wyrich, R., Westhoff, P., Hense, K., Schnarrenberger, C., and Martin, W. (1995) *Plant Mol. Biol*. **29,** 1279-1291

Van de Peer, Y., and De Wachter, R. (1993) *Comput. Applic. Biosci.* **9,** 177-182

Martin, W., and Schnarrenberger, C. (1997) *Curr. Genet.* **32,** 1-18

# Poster S4

## THE BETALAINS : ELUSIVE PIGMENTS AND THEIR POTENTIAL AS *IN VIVO* MARKERS

*Hinz, U., Zaiko, M, Christinet, L. and Zryd, J.-P.*

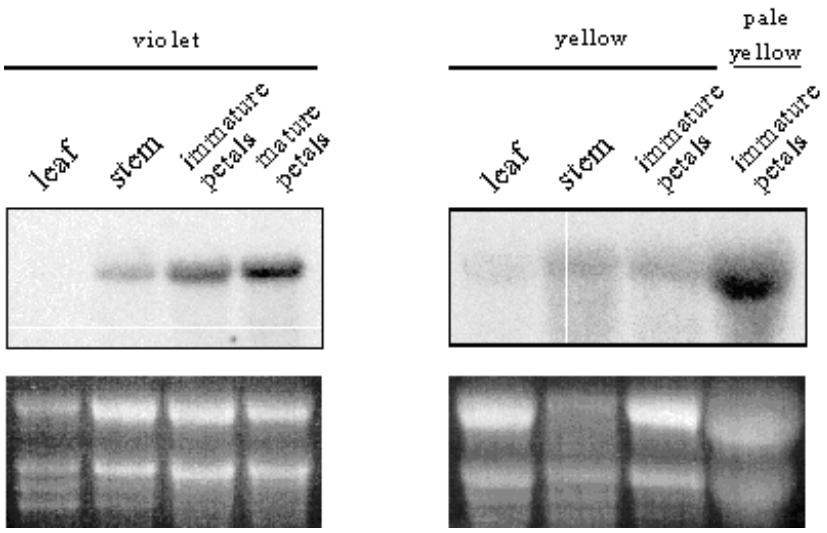
Université de Lausanne ; IE-PC, 1015 Lausanne

Betalains are a class of yellow or violet vacuolar pigments characteristic for plants of the order Caryophyllales and also a few fungal species, e.g. *Amanita muscaria* and *Hygrocybe species.* Their structure has been elucidated and the pathway is well characterised as to the chemical intermediates.

Betalain biosynthesis is subject to complex regulation and the pigments accumulate only in certain tissues and at specific stages of development. Genetic analysis of betalain biosynthesis in the ornamental plant *Portulaca grandiflora* indicated that three *loci* were sufficient: **C** converts DOPA to betalamic acid, **R** is necessary for the accumulation of violet betacyanins and **I** inhibits the accumulation of betaxanthins.

Betalains are highly colored and can be seen easily in single cells. This makes them good candidates for use as *in vivo* markers; they could serve to identify transformed cells in biolistic transformation or they could be used in classical promoter studies. The gene coding for DOPA-dioxygenase has been cloned from *A. muscaria* and has been shown to complement betalain biosynthesis in *P. grandiflora*, but we still have to identify its plant counterpart and the other genes involved in this pathway. Except for the DOPA-dioxygenase their exact mode of action remains mysterious. *In vitro* the tyrosinase converts tyrosine to DOPA, but then oxidises it further to DOPA-quinone, which then can give cyclo-DOPA. *In vivo* the situation clearly is more complex: by means unknown at least part of the DOPA is protected against further oxidation, otherwise there would not be enough cytoplasmic DOPA for conversion to betalamic acid and besides all betalain-producing tissues should contain at least some betacyanins. This is in clear contradiction to the existence of betalain-producing plants with pure yellow petals. If a tyrosinase is sufficient to give cyclo-DOPA, then what is the function of gene **R**? Could it be another tyrosinase in another cellular compartment? And if betaxanthins arise by the spontaneous reaction between betalamic acid and amino acids, how can gene **I** inhibit the formation of betaxanthins, and how can one explain that plants accumulate only a limited spectrum of betaxanthins?

Wishing to identify a maximum of betalain genes we opted for a molecular approach and made subtraction libraries to clone genes specifically expressed in colored petals, but absent in white petals. A first subtraction between yellow and white petals yielded several potentially interesting clones, three of which (V33, L13 and L6) showed an expression pattern corresponding to that expected for a betalain gene. They are highly expressed in colored petals and violet stems, but not detectable in tissues without betalains as exemplified on this Northern blot with clone L13.




Another subtraction has been made between violet and white petals. Out of 900 clones ca 5% corresponded to V33 and were eliminated. Of the remaining clones a first analysis by slot blot indicated that 10-20% showed violet-specific expression. These clones are currently being analysed and we hope to obtain clones for **C**, **R** and **I**, but also for enzymes that modify betalains (glycosylation) and regulatory genes, although these latter genes might be expected to be present in low copy number and therefore hard to detect. Apart from genes directly involved in betalain biosynthesis we expect to find a number of genes not directly coding for betalain synthesis, but indirectly related to betalain accumulation: transport proteins for the export of tyrosine or DOPA from the plastids, vacuolar transporters for the import of betalains into the vacuole, etc. Obtaining an overview of the number and type of genes co-regulated with betalain pigments will be highly interesting by itself and can serve as an example for other biochemical events.

# Poster S5

## VESICULAR TRAFFICKING IN *ARABIDOPSIS THALIANA*: CHARACTERIZATION OF THE T-SNARE HOMOLOGUE ATSNAP33

*Peter Wick, Xavier Gansel[1] and Liliane Sticher*

Department of Biology, Unit Plant Biology, University of Fribourg, 3, rte A. Gockel, 1700 Fribourg, Switzerland

[1] present address: Biochimie et Physiologie moléculaire des Plantes, Place Viala, 34060 Montpellier, France

### Introduction

In plants, secretion is involved in numerous processes including cell growth, cell wall formation, pollen tube growth, xylem differentiation and plant defense against pathogens (Battey et al., 1999). Transport along the secretory pathway requires budding of cargo vesicles from a donor membrane and fusion with an acceptor membrane. Maintenance of the cellular compartmentalization is essential for the function of the cell and requires correct transport between the compartments allowing fusion of vesicles only with the appropriate membrane. Exocytosis is the fusion of vesicles with the plasma membrane releasing the content of the vesicle outside the plasma membrane. The SNARE model states that vesicle fusion is mediated by the interaction between membrane proteins on the vesicle (v-SNAREs: vesicle SNAP receptors) and on the target membrane (t-SNAREs) (Rothman, 1996). In animal cells and yeast, different t- and v-SNAREs have been found to be distributed in different compartments of the secretory pathway (Hay and Scheller, 1997). At the synapse, exocytosis of vesicles containing neurotransmitters involves the association of a v-SNARE, synaptobrevin and 2 t-SNAREs, syntaxin1 and SNAP-25 (synaptosomal associated protein of 25 kDa). These SNAREs form a complex that binds proteins required for general traffic, ?-SNAP (soluble NSF-attachment protein) and NSF (N-ethylmaleimide-sensitive factor).

Although little is known on the molecular mechanisms of vesicle fusion in plants, several homologues of SNAREs have been characterized suggesting that the SNARE model also applies to plant cells (Sanderfoot and Raikhel, 1999).

### Results and discussion

We isolated a cDNA from *Arabidopsis thaliana* called *AtSNAP33* encoding a protein which shows homology to the neuronal t-SNARE, SNAP-25, and to the non neuronal homologues syndet, SNAP-23 and yeast sec 9. Using a yeast two hybrid system to find proteins which interact with AtSNAP33, two novel *A. thaliana* cDNAs encoding syntaxins were isolated. We postulate that AtSNAP33 is a t-SNARE involved in exocytosis.

### AtSNAP33 is an integral membrane protein

AtSNAP33 was localized at the plasma membrane by immunoelectron microscopy. To confirm that it is a membrane protein, differential centrifugation was performed. AtSNAP33 was in the pellet after centrifugation at 8000 g and 100000 g. No AtSNAP33 was found in the supernatant after centrifugation at 100000 g indicating that AtSNAP33 was associated with the microsomal fraction. AtSNAP33 was not solubilized from the membrane by treatment with 2M urea, 1M NaCl, or 0.1 M $Na_2CO_3$ pH 11 which solubilize peripheral membrane proteins. It was solubilized by treatment with 1% SDS. Thus AtSNAP33 is an integral membrane protein.

### The expression of AtSNAP33 is induced after infection with *Peronospora parasitica*

The kinetic of expression of *AtSNAP33* and *PR-1* was analysed after inoculation of *A. thaliana* accession Col-0 with the downy mildew pathogen *Peronospora parasitica* isolate NOCO and EMWA. The isolate NOCO forms a compatible interaction and the isolate EMWA an incompatible interaction with *A. thaliana* accession Columbia. In the compatible interaction with the isolate NOCO, the hyphae grew into the plant tissues, forming numerous haustoria and no symptoms were visible before 7 days when the conodiophores bearing the asexual conidia grew out of the stomata. In the incompatible interaction with the isolate EMWA, the hyphae grew for 3 days forming haustoria. Then growth of the fungus stopped and trailing necroses of host cells along the length of the hyphae were observed.

There was an increase of *AtSNAP33* transcripts 2 days and 3 days after inoculation with the isolate EMWA. The expression was back to control levels after 4 days. In contrast, the level of *AtSNAP33* transcripts increased 4 days post inoculation (dpi) and remained high until 7 dpi with *P. parasitica* isolate NOCO.

In the incompatible interaction with isolate EMWA, the level of *PR-1* transcripts increased after 3 days and stayed high until 7 dpi. In the compatible interaction with the isolate NOCO, the level of *PR-1* transcripts increased 4 dpi and stayed high until 7 dpi.

The differences in the expression of *AtSNAP33* and *PR-1* suggest that the expression of the 2 genes is regulated by different signalling pathways. The increased expression of *AtSNAP33* after pathogen attack may be related to the increased secretion of host defense compounds including PR proteins and to the formation of the host membrane around haustoria.

# Poster S6

## EXPRESSION OF A TAGGED PLANT PROTEIN IN THE YEAST *PICHIA PASTORIS* FOR STRUCTURE-FUNCTION ANALYSIS: SUCROSE: FRUCTAN 6-FRUCTOSYLTRANSFERASE (6-SFT), A KEY ENZYME OF FRUCTAN SYNTHESIS IN BARLEY

*Alain Denis Meyer, Denise Altenbach, Marcel Lüscher, Urs Hochstrasser, Thomas Boller and Andres Wiemken*

Department of Botany, University of Basel, Hebelstresse 1, 4056 Basel, Switzerland

Fructans (linear or branched polymers of â-fructosyl units linked to a terminal glucose unit) are the second-most prevalent reserve carbohydrates in plants: about 15% of the species store fructan, either in addition or as an alternative to starch. Among those are various cereals, forage grasses, and vegetables. Being part of the fundamental carbon metabolism, and because fructan has many potential applications in food industry (emulsifiers, low-calorie health food etc.), fructans have received much attention in the last decade (see Heyer *et al.*, 1999, for a recent review).

The enzymes involved in fructan synthesis are highly homologous to acid invertases and share common features such as subcellular localization (vacuolar), post-translational processing (cleavage into subunits) and several highly conserved domains in their peptide sequences. Therefore, it appears that the fructan synthesis enzymes evolved through slight modifications from acid invertases, presumably several times during evolution as judged by the distribution of fructan-containing plant genera and species.

In earlier studies of our group, barley has proven to be a particularly suitable model system to study fructan synthesis. In excised barley leaves put under continuous illumination, the induction of a specific sucrose:sucrose 1-fructosyltransferase (1-SST, producing 1-kestose) and of a specific sucrose:sucrose/fructan 6-fructosyltransferase (6-SST/SFT, producing 6-kestose or the dp4 fructan bifurcose) occurred concomitantly with the accumulation of fructan in the induced leaves (Wagner *et al.*, 1986; Wagner and Wiemken, 1987, 1989; Obenland *et al.*, 1991, 1993; Simmen *et al.*, 1993). This clarified that specific enzymes are involved in fructan biosynthesis and that fructan accumulation cannot be accounted for by secondary activities of acid invertases. Later, evidence was obtained that the 6-SFT is a key enzyme for the formation of branched fructans (graminans) from sucrose. Analysis of the substrate specificities of purified 1-SST, 6-SST/SFT and of two purified acid invertases gave strong indication for fructan originating through the consecutive action of 1-SST and 6-SFT in barley leaves (Obenland *et al.*, 1993; Simmen *et al.*, 1993; Duchateau *et al.*, 1995).

After the success in cloning the 6-SST/SFT from barley, functional expression in *Nicotiana plumbaginifolia* protoplasts provided the first molecular evidence for a specific fructan-synthesizing enzyme (Sprenger et al., 1995). Thereafter, using the same clone, we established a yeast (*Pichia pastoris*) expression system where the enzyme is secreted into the culture medium (Hochstrasser et al., 1998). This system appears to be particularly suitable for structure-function analyses as *Pichia pastoris* does not produce extracellular invertases under normal conditions. This is a considerable advantage over plant expression systems where the invertase activities interfere with the analyses

Our final goal is to determine the structural requirements of enzymes belonging to this invertase/ fructosyltransferase family in order to be able to assign certain structural features to particular activities. Surprisingly, the 6-SFT enzyme produced in *Pichia* showed an additional 1-SST activity (producing 1-kestose) when incubated with sucrose alone as a substrate. This activity was absent when the purified barley 6-SFT was analyzed (Hochstrasser *et al.* 1998). We constructed also a tagged version of the 6-SFT, with a *myc* epitope and a C-terminal poly-histidine peptide for expression in *Pichia* and subsequent purification and detection. The key activities of the new, tagged 6-SFT are summarized below in comparison with the non-tagged *Pichia* produced enzyme and earlier data with the enzyme purified from barley. The tagged enzyme showed a higher relative 6-SST activity than the non-tagged recombinant 6-SFT.

| | | Activities [% of total fructosyl transfer] | | | |
|---|---|---|---|---|---|
| 6-SFT enzyme | Substrate | Invertase | 1-SST | 6-SST | 6-SFT |
| Purified from barley[1)] | | 80 | <0.1 | 20 | - |
| Recombinant untagged[2)] | Sucrose | 89 | 5 | 6 | - |
| Recombinant tagged | | 83 | 8 | 9 | - |
| Purified from barley[1)] | Sucrose | 20 | - | 5 | 80 |
| Recombinant untagged[2)] | + | 70 | - | 4 | 26 |
| Recombinant tagged | 1-kestose | 74 | - | 11 | 15 |

1) from Duchateau *et al.*, 1995 - 2) from Hochstrasser *et al.*, 1998

Possible explanations for the appearance of the additional activity are differences in post-translational processing between barley and *Pichia* (glycosylation, subunit cleavage) and/or chemical factors affecting activity in the extracts. The activity shift of the tagged 6-SFT in favor of higher 6-SST activity when compared to the non-tagged version may be due to conformational changes or altered charge distributions caused by the C-terminal tags. We predict that the structural changes we plan to introduce into the 6-SFT protein *via* site-directed mutagenesis will unravel determining elements of the different enzymatic activities.

References:

Duchateau N., Bortlik K.H., Simmen U., Wiemken A., Bancal P. (1995), Plant Physiol. 107, 1249-1255

Heyer A.G, Lloyd J., Kossmann J. (1999), Curr. Opinion Biotechnol. 10, 169-174

Hochstrasser U., Lüscher M., De Virgilio C., Boller T., Wiemken A. (1998), FEBS Lett. 440, 356-360

Obenland D., Simmen U., Boller T., Wiemken A. (1991), Plant Physiol. 97, 811-813

Obenland D., Simmen U., Boller T., Wiemken A. (1993), Plant Physiol. 101, 1331-1339

Sprenger N., Bortlik K. H., Brandt A., Boller T., Wiemken A. (1995) Proc. Natl. Acad. Sci.92, 11652-11656

Simmen U., Obenland D., Boller T., Wiemken A. (1993), Plant Physiol. 101, 459-468

Wagner W., Wiemken A., Matile P. (1986), Plant Physiol. 81, 444-447

Wagner W., Wiemken A. (1987), Plant Physiol. 85, 706-710