Ph.D. thesis

# Study of proline accumulation and transcriptional regulation of genes involved in this process in *Arabidopsis thaliana*

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## Background

Environmental factors that impose water-deficit stress, such as drought, salinity or extreme temperature, place major limits on plant productivity. Upon exposure to osmotic stress, plants exhibit a wide range of responses at molecular, cellular and whole-plant levels. These include, for example morphological and developmental changes (e.g. shortening of life cycle, inhibition of shoot growth and enhancement of root growth), adjustment in ion transport and metabolic changes (e.g. carbon metabolism, the synthesis of compatible solutes). Under osmotic stress condition characteristic up- and down-regulation of gene expression can be observed. These transcriptional changes may be regulated directly by stress conditions or may result from secondary stresses and/or injury responses. A complex series of yet not clearly clarified signal transduction events lead to changing in gene expression pattern. One of the signals involved is abscisic acid (ABA).

Genes which are up-regulated by water stress can be classified into functional categories. These gene products are involved in detoxification (e.g. free radical scanging enzymes), transport (aquaporins), transcription, signal transduction, protecting of cytoplasmic macromolecules (LEA proteins), protein degradation, metabolism (e.g. osmolyte biosynthesis, photosynthesis).

Increased concentration of charged molecules in the cytosol may change the hydration sphere of macromolecules and thus affects their conformation or charge interactions. Compatible osmolytes are important for maintaining the conformation of macromolecules. Osmolytes were originally thought to function mainly in osmotic adjustment by lowering cellular osmotic potential to facilitate water absorption and restore intracellular salt concentrations. Common osmolytes include sugars, polyols and amino acids or their derivatives. These compounds appear to have little interference in the function of macromolecules, even at very high concentrations.

Proline is one of the osmoprotective molecules which accumulates in many organisms, including bacteria, fungi, invertebrates and plants in response to water stress and salinity. Genetic studies in prokaryotes demonstrate that proline is an essential compatible osmolyte because proline overproduction in bacteria confers acquired osmotic stress tolerance. In addition to biophysical protective effects of free proline, it has been proposed that a stress-induced increase in the transfer of reducing equivalents into proline by $\Delta$-$^{1}$-pyrroline-5-carboxylate synthetase and P5C reductase may be a protective mechanism whereby many species ameliorate shifts in cellular redox potential which accompany all biotic and abiotic

stresses. An additional benefit of the replenishment of $NADP^+$ supply by proline synthesis may be to support redox cycling. This was proposed to be important in plant antioxidant defence mechanisms during stress. A signal derived from the proline biosynthetic and catabolic pathways, possibly the redox potential of the pyridine nucleotide pools, may control gene expression in response to osmotic stress. Furthermore proline is thought to function as free radical scavenger. Proline degradation can serve as carbon, nitrogen and energy source upon relief from stress. In higher plants proline can be synthesized either from glutamate or ornithine. The glutamate pathway is dominant under osmotic stress conditions. In the glutamate pathway the bifunctional $\Delta$-$^1$-pyrroline-5-carboxylate synthetase phosphorylates and reduces the glutamate to glutamyl-5-semialdehyde (GSA) which is spontaneously converted to pyrroline-5-carboxylate (P5C). P5C is reduced to proline by the pyrroline-5-carboxylate reductase (P5CR). The rate limiting step is the $\gamma$-glutamyl kinase activity of the P5CS which is feed back regulated by proline. Degradation of proline takes place via oxydation to P5C by proline dehydrogenase (PDH) and subsequently to L-glutamic acid by P5C dehydrogenase. The proline accumulation during osmotic stress is the result of up-regulation of biosynthetic and down-regulation of degradation processes.

There is growing awareness on interactions between different environmental and hormone signals that predict a network of signal transduction pathways controlling responses to environmental stimuli, growth factors or developmental signals. Plant responses to osmotic stress should coordinate diverse functions, therefore regulation of responses requires multiple interacting signalling cascades that are able to detect and transmit numerous external and internal signals. Crosstalk between sugar and hormone signals have been described, e.g. the *prl1* mutant of *Arabidopsis* is hypersensitive to sugar, cold, and several plant hormones including ABA, ethylene, auxin. This mutant shows transcriptional derepression of genes controlled by light, sugar repression, pathogen and environmental stress responses. The pleiotrop effect of mutation suggests, that the WD 40 repeat regulator protein encoded by the wild type *PRL1* gene is essential for coordination of multiple signalling pathways.

Positive effect of light on proline accumulation has been described. Daily oscillation of the *AtP5CS1* gene activity and fluctuation in the proline content in *Arabidopsis* have been reported recently, indicating that light-dependent signals may affect *P5CS* expression. In higher plants brassinosteroids are naturally occuring polyhydroxy steroids that are able to control wide range of biological functions. These compounds repress light responses, promote plant development and cell elongation. Brassinosteroids have been implicated in controlling responses to various environmental factors, such as cold, salt, injury or high temperature.

Numerous data suggest that crosstalk exists between light and steroid signals, glucose catabolic repression and hormone action, which influences stress responses in higher plants.

Aim of our study

At the beginning of our work the gene encoding P5CS was unknown in *Arabidopsis thaliana,* therefore our goals were:

- Isolation of *P5CS* gene(s)
- Mapping and sequence analysis of the *P5CS* gene(s)
- Gene expression study:
    - Tissue specificity
    - Stress inducibility
    - Hormonal regulation
    - „Crosstalk" with other factors
- Promoter analysis

Methods

- Molecular cloning techniques
- Generating transgenic *Arabidopsis*
- Qualitative and quantitative GUS assays
- Total RNA isolation from plant
- Northern hybridization
- Proline determination

Results and discussion

1. We pointed out that 200 mM NaCl-, 200 mM glucose- and 200 mM mannitol-treatments increased the free proline level and cold treatment had little effect on it. Among hormons only the abscisic acid could significantly enhance the proline accumulation. The NaCl- and ABA-induced proline accumulation could be inhibited by five days of dark adaptation.

2. We could isolate two distinct cDNAs coding for $\Delta$-$^{1}$-pyrroline-5-carboxylate synthetase (P5CS) In comparison with the *AtP5CS1* cDNA encoding the P5CS1 enzyme of 717 amino acids (77.7 kDa) the *AtP5CS2* cDNA consisted of 726 codons coding for the P5CS2 isoenzyme (78.8 kDa). The two *AtP5CS* cDNAs shared on overall identity of 82 %, yielding an amino acid similarity of 93 % between the two isoenzymes. Nonetheless, the 5' and 3' non-coding regions of *AtP5CS* cDNAs displayed only 54 % and 53 % sequence identity, respectively, providing useful gene specific probes. The multiple alignment analysis indicated the existence of conserved kinase and dehydrogenase domains, putative ATP and NADPH-binding sites, and leucine-rich region. Furthermore two amino acid residues implicated in feed-back inhibition of the *Vigna aconitifolia* P5CS enzyme by proline are present in both *AtP5CS* sequences.

3. Northern hybridization with gene specific probes detected significant levels of *AtP5CS1* mRNA in roots, stem, leaves and flowers. In comparison, the levels of *AtP5CS2* mRNA were much lower in most plant organs. However, in actively dividing calli and cell suspension cultures the *AtP5CS1* transcript was undetectable, whereas the *AtP5CS2* mRNA represented an abundant transcript. These results were proved by analyses of transgenic *Arabidopsis* plants carrying *AtP5CS1::GUS* and *AtP5CS2::GUS* reporter gene constructs.

4. Both *AtP5CS* genes can be induced by dehydration, NaCl- and ABA-treatments, but with different kinetics. Generally, the induction of *AtP5CS1* gene is faster and stronger than that of *AtP5CS2* gene.

5. The induction of *AtP5CS* mRNA synthesis in salt-treated *Arabidopsis* seedlings follows roughly an exponential curve. Our data show that the fast linear phase of induction is not inhibited by cycloheximide, thus probably represents an immediate early response. The inhibition of protein synthesis by cycloheximide prevents the second, slower phase of salt-induction. The protein synthesis is probably required for continuous accumulation and maintenance of induced levels of *AtP5CS* mRNAs during salt stress.

*6.* NaCl-induced accumulation of *AtP5CS* mRNAs was monitored in mutant *Arabidopsis* lines where the mutations caused ABA-deficiency or ABA-insensitivity. In ABA-deficient *aba1-1* mutant, NaCl-treatment failed to induce any increase of *AtP5CS* transcript levels, demonstrating that ABA is absolutely essential as signaling molecule for salt-induced activation of *AtP5CS1* and *AtP5CS2* genes. Morever not only ABA biosynthesis, but also some steps in ABA (and possibly auxin) signalling, that are impaired in the *abi1-1* and *axr2* mutants, are implicated in the control of basic level and salt-induced accumulation of both *AtP5CS* transcripts in *Arabidopsis.*

7. We could prove that the inhibitory effect of dark adaptation on ABA- and NaCl-induced proline accumulation is due to up-regulation of *P5CS1* and down-regulation of *PDH* genes in *Arabidopsis.*

8. We presented that as dark adaptation, 24-epibrassinolide treatment could inhibit the *AtP5CS1* mRNA accumulation induced by NaCl or ABA. The inhibitory effect of 24-epibrassinolide is less pronounced in the case of *AtP5CS2* gene (only in leaves). *PDH* gene expression is not influenced by epibrassinolide.

9. 1.6-1.8-fold increase of total *AtP5CS1* transcript levels was observed in response to ABA and salt induction in seedlings that carried a mutation in the *pleiotropic regulatory locus 1* (*prl1*) causing hypersensitivity to ABA. The ABA- and NaCl-treated *prl1* mutant plants had higher free proline content than wild type because of increased *AtP5CS1* transcription. In ABA-treated, brassinosteroid-deficient *det2* mutant increased *AtP5CS1* mRNA and proline accumulation was observed. *det2* and *prl1* mutations did not alleviate the inhibitory effect of 24-epibrassinolide

pretreatment on *AtP5CS1* transcript level. *AtP5CS2* transcription is slightly increased in *det2* mutant and can be repressed by 24-epibrassinolide. *PDH* transcript level was slightly decreased by epibrassinolide in wild type plants. In *det2* and *prl1* mutants the epibrassinolide repression was weaker on *PDH* transcript level.

10. We proved that in *prl1* mutant increased activity of *AtP5CS1::GUS* reporter gene construct can be found which is partially inhibited by 24-epibrassinolide. *prl1* mutation had little effect on *AtP5CS2::GUS* reporter gene construct.

Finally, we can conclude that in contrast to previous literature data the bifunctional enzyme which catalyzes the rate limiting step of proline biosynthesis is encoded by two genes with different tissue specific expression and transcriptional regulation in *Arabidopsis thaliana*. Morever transcriptional regulations of these *AtP5CS* genes have major role in proline accumulation during osmotic stress.

Publications:

Publications involved in thesis:

Strizhov, N., **Ábrahám**, E., Ökrész, L., Blickling, S., Zilberstein, A., Schell, J., Koncz, Cs., Szabados, L. (1997) Differential expression of two *P5CS* genes controlling proline accumulation during salt-stress requires ABA and is regulated by *ABA1, ABI1* and *AXR2* in *Arabidopsis. Plant Journal* 12(3): 557-569

**Ábrahám, E**., Rigó., G., Székely, G., Nagy, R., Koncz, Cs., Szabados, L. (2003) Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in *Arabidopsis. Plant Molecular Biology* 51: 363-372.

Other publications:

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Szabados, L., **Abraham E.**, Okresz, L., Strizhov, N., Zilberstein, A., Schell, J., Koncz, C. (1998) Structure, function and regulation of *AtP5CS* genes in *Arabidopsis*. *Biotechnology & Biotech Equipment.* 12: 3-10.

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