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Differential expression of two *P5CS* genes controlling proline accumulation during salt-stress requires ABA and is regulated by *ABA1*, *ABI1* and *AXR2* in *Arabidopsis*

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Summary

Proline is a common compatible osmolyte in higher plants. Proline accumulation in response to water stress and salinity is preceded by a rapid increase of the mRNA level of Δ^1-pyrroline-5-carboxylate synthase (P5CS) controlling the rate-limiting step of glutamate-derived proline biosynthesis. P5CS is encoded by two differentially regulated genes in *Arabidopsis*. Gene *AtP5CS1* mapped to chromosome 2-78.5 is expressed in most plant organs, but silent in dividing cells. Gene *AtP5CS2* located close to marker m457 on chromosome 3–101.3 contributes 20–40% of total P5CS mRNA in plant tissues, but is solely responsible for the synthesis of abundant P5CS mRNA in rapidly dividing cell cultures. Accumulation of *AtP5CS* transcripts is regulated in a tissue specific manner and inducible by drought, salinity, ABA, and to a lesser extent by auxin. Induction of *AtP5CS1* mRNA accumulation in salt-treated seedlings involves an immediate early transcriptional response regulated by ABA signalling that is not inhibited by cycloheximide, but abolished by the deficiency of ABA biosynthesis in the *aba1 Arabidopsis* mutant. However, inhibition of protein synthesis by cycloheximide prevents the induction of *AtP5CS2* mRNA accumulation, and blocks further increase of *AtP5CS1* mRNA levels during the second, slow phase of salt-induction. Mutations *abi1* and *axr2*, affecting ABA-perception in *Arabidopsis*, reduce the accumulation of both *AtP5CS* mRNAs during salt-stress, whereas ABA-signalling functions defined by the *abi2* and *abi3* mutations have no effect on salt-induction of the *AtP5CS* genes.

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Introduction

Proline is one of the osmoprotecting molecules (osmolytes) which accumulates in many organisms, including bacteria, fungi, algae, invertebrates and plants in response to water stress and salinity (for review see Csonka and Hanson, 1991; Delauney and Verma, 1993; Hanson and Hitz, 1982; Yoshiba *et al.*, 1995). Genetic studies in prokaryotes demonstrate that proline is an essential compatible osmolyte because proline overproduction in bacteria confers acquired osmotic stress tolerance (Csonka, 1989; Mahan and Csonka, 1983). Correlations between proline accumulation and osmotic stress responses indicate that proline also plays a role as osmoprotectant in higher plants (Chiang and Dandekar, 1995; Martinez *et al.*, 1995; Ober and Sharp, 1994; Serrano and Glaxiola, 1994; Thomas *et al.*, 1992). Because drought and salinity represent severe agricultural constraints world-wide, studies on the regulation of proline biosynthesis in plants received attention (Daugherty *et al.*, 1994; Ingram and Bartels, 1996).

In higher plants proline is produced from either glutamate or ornithine. Biochemical studies indicate that the glutamate pathway is the dominant stress-controlled pathway and interlinked with the regulation of *de novo* purine biosynthesis (Delauney and Verma, 1993). Generation of $NADP^+$ by the glutamate pathway stimulates the production of purine precursor ribose-5-phosphate, modulating the biosynthesis of ureides required for nitrogen storage and transport, particularly in nitrogen-fixing nodules of legumes (Kohl *et al.*, 1988). In the glutamate pathway of proline biosynthesis, glutamic acid is phosphorylated and reduced to glutamyl-5-semialdehyde (G5SA) in plants by a bifunctional Δ^1-pyrroline-5-carboxylate synthase (P5CS), which possesses both γ-glutamyl kinase (γ-GK) and glutamic-γ-semialdehyde dehydrogenase (GSA-DH) activities (Hu *et al.*, 1992). Proline is synthesized from G5SA via pyrroline-5-carboxylate (P5C) by the Δ^1-pyrroline-5-carboxylate reductase (P5CR) enzyme (Delauney and Verma, 1990, 1993). Cell fractionation studies detected P5CR activity in both cytoplasm and chloroplast, indicating that proline biosynthesis may occur in different subcellular compartments (Rayapati *et al.*, 1989; Szoke *et al.*, 1992). As is the case in bacteria, proline controls the γ-GK activity of P5CS also in plants by feed-back inhibition, which probably represents the rate-limiting step in proline biosynthesis (Csonka, 1989; Zhang *et al.*, 1995).

Genes and cDNAs coding for P5CS and P5CR have been isolated from different plant species, including *Arabidopsis*

thaliana, pea, and *Vigna aconitifolia* (Delauney and Verma, 1990; Hu *et al.*, 1992; Savouré *et al.*, 1995; Verbruggen *et al.*, 1993; Williamson and Slocum, 1992; Yoshiba *et al.*, 1995). In addition, proline accumulation in *Arabidopsis* was demonstrated to follow faithfully the transcriptional induction of the P5CS gene in response to hormonal and environmental stress stimuli, such as abscisic acid (ABA), drought, and salinity (Yoshiba *et al.*, 1995). Because expression of the P5CR gene is not affected significantly by these stress stimuli in *Arabidopsis*, the stress-induced accumulation of proline appears to be regulated by the expression of *P5CS* gene (Delauney and Verma, 1990; Savouré *et al.*, 1995; Yoshiba *et al.*, 1995). In fact, overexpression of a mothbean *P5CS* enzyme in transgenic tobacco has been reported to result in elevated proline content and altered drought tolerance responses (Kavi Kishor *et al.*, 1995).

Genetic analysis of the regulation of amino acid biosynthesis pathways in higher plants faces the problem that most metabolic steps involve multiple isoenzymes encoded by small families of differentially regulated genes (Coruzzi, 1991; Pickett and Meeks-Wagner, 1995; Rose and Last, 1994). Therefore, previous reports emphasizing the finding that P5CS is encoded by a single copy gene in *Arabidopsis* (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995) are of particular importance because this fact could facilitate the isolation of P5CS mutants in plants. The data presented here show, however, that such a mutational analysis may not be so simple, because P5CS is in fact encoded by two differentially regulated genes in *Arabidopsis*. Expression of the known *AtP5CS1* gene occurs in differentiated tissues, but cannot be detected in dividing cell cultures in the absence of stress stimuli. In contrast, the newly identified *AtP5CS2* gene is active in dividing cells, as well as transcribed in other plant tissues albeit at lower levels than *AtP5CS1*. In spite of quantitative differences in their steady-state mRNA levels, both *AtP5CS* genes are similarly induced by drought, salt and ABA, as well as down-regulated by the *abi1* and *axr2* mutations, affecting ABA and auxin perception, respectively. Proline biosynthesis thus appears to be redundantly controlled by two *P5CS* genes in *Arabidopsis*, one of which (*AtP5CS2*) is probably involved in the osmoprotection of dividing meristemic tissues.

Results

Δ^1-pyrroline-5-carboxylate synthase (P5CS) is encoded by two genes in Arabidopsis

A segment of *Vigna P5CS* cDNA, sharing significant sequence similarity (between positions 1954 and 2037; Hu *et al.*, 1992) with the *E. coli proA* gene (Deutsch *et al.*, 1984), was used as an oligonucleotide probe to isolate *P5CS* clones from *Arabidopsis* cDNA libraries prepared from seedlings and cell suspension cultures. Nucleotide sequence analysis of six clones (EMBL/Genbank X86777) from the seedling cDNA library revealed complete sequence identity with the *P5CS* cDNA from Savouré *et al* (1995) and some mismatches in comparison with a similar sequence reported by Yoshiba *et al.* (1995). In contrast, six clones derived from the cell suspension cDNA library carried segments of a novel *P5CS* cDNA, which was converted to a full-length cDNA clone by 5′-RACE PCR amplification (Frohman *et al.*, 1988, EMBL/Genbank Y09355). In comparison with the known *AtP5CS1* cDNA, encoding the P5CS1 enzyme of 717 amino acids (77.7 kDa), the open reading frame of new *AtP5CS2* cDNA consisted of 726 codons coding for the P5CS2 isoenzyme of 78.8 kDa. The two *AtP5CS* cDNAs shared an 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, respectively, only 54% and 53% sequence identity, providing useful gene specific probes.

A comparison of γ-GK and GSA-DH sequences from *E.coli* (Deutsch *et al.*, 1984) and *Serratia marcescens* (Omori *et al.*, 1992) with P5CS sequences from *Arabidopsis* (Savouré *et al.*, 1995) and *Vigna* (Hu *et al.*, 1992) indicated an overall conservation of kinase and dehydrogenase domains, putative ATP and NADPH-binding sites, and leucine-rich regions (Savouré *et al.*, 1995), and showed that two amino acid residues implicated in feed-back inhibition of the *Vigna* P5CS enzyme by proline (Zhang *et al.*, 1995) are present in both *AtP5CS* sequences (Figure 1). In contrast, amino acid residues involved in feed-back inhibition of bacterial γ-GK enzymes (Csonka and Hanson, 1991, Csonka *et al.*, 1988; Omori *et al.*, 1992) were not found to be conserved in the *Arabidopsis* P5CS sequences, which also lacked typical signal peptides for potential chloroplast transport.

Genetic mapping and characterization of the AtP5CS2 *gene*

Southern hybridization of DNAs from *Arabidopsis* ecotypes Columbia and Landsberg, using either the *AtP5CS* cDNAs or their divergent 3′-ends as gene specific probes, detected clear differences in the hybridization pattern of *AtP5CS1* and *AtP5CS2* genes (Figure 2c). Using the cDNA probes, a restriction fragment length polymorphism (RFLP) was found with *Kpn*I for *AtP5CS2*, and a previously reported *Eco*RI RFLP for *AtP5CS1* was confirmed (Savouré *et al.*, 1995). Both genes were mapped to yeast artificial chromosome (YAC) clones using gene specific probes. The *AtP5CS1* probe hybridized to overlapping YACs CIC10A6, CIC10A12 and CIC9E4, but not to the neighbouring clones CIC10F12, CICG9 and CIC11C8 (Figure 2a and b). This allowed us to refine the previous mapping data (Savouré

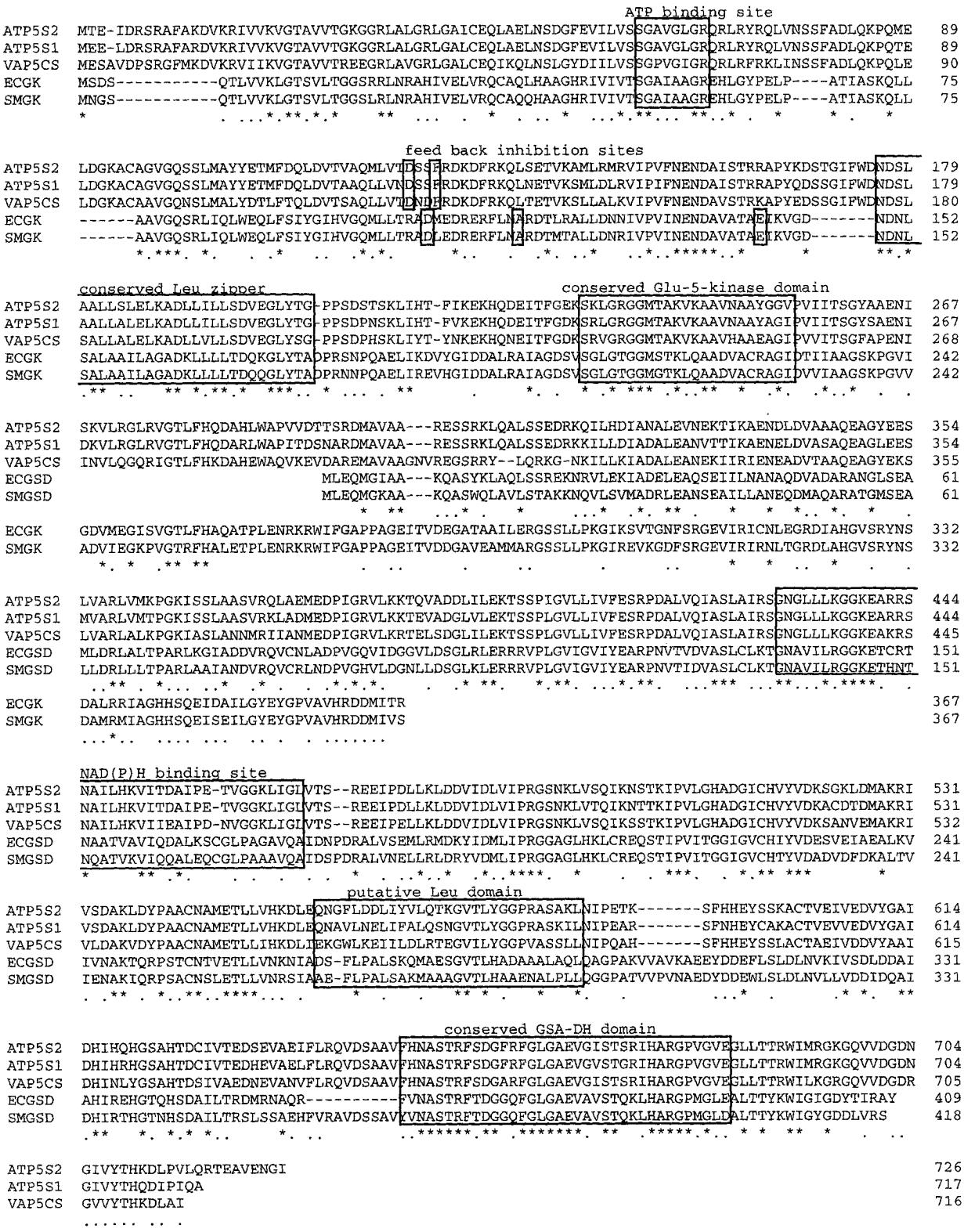
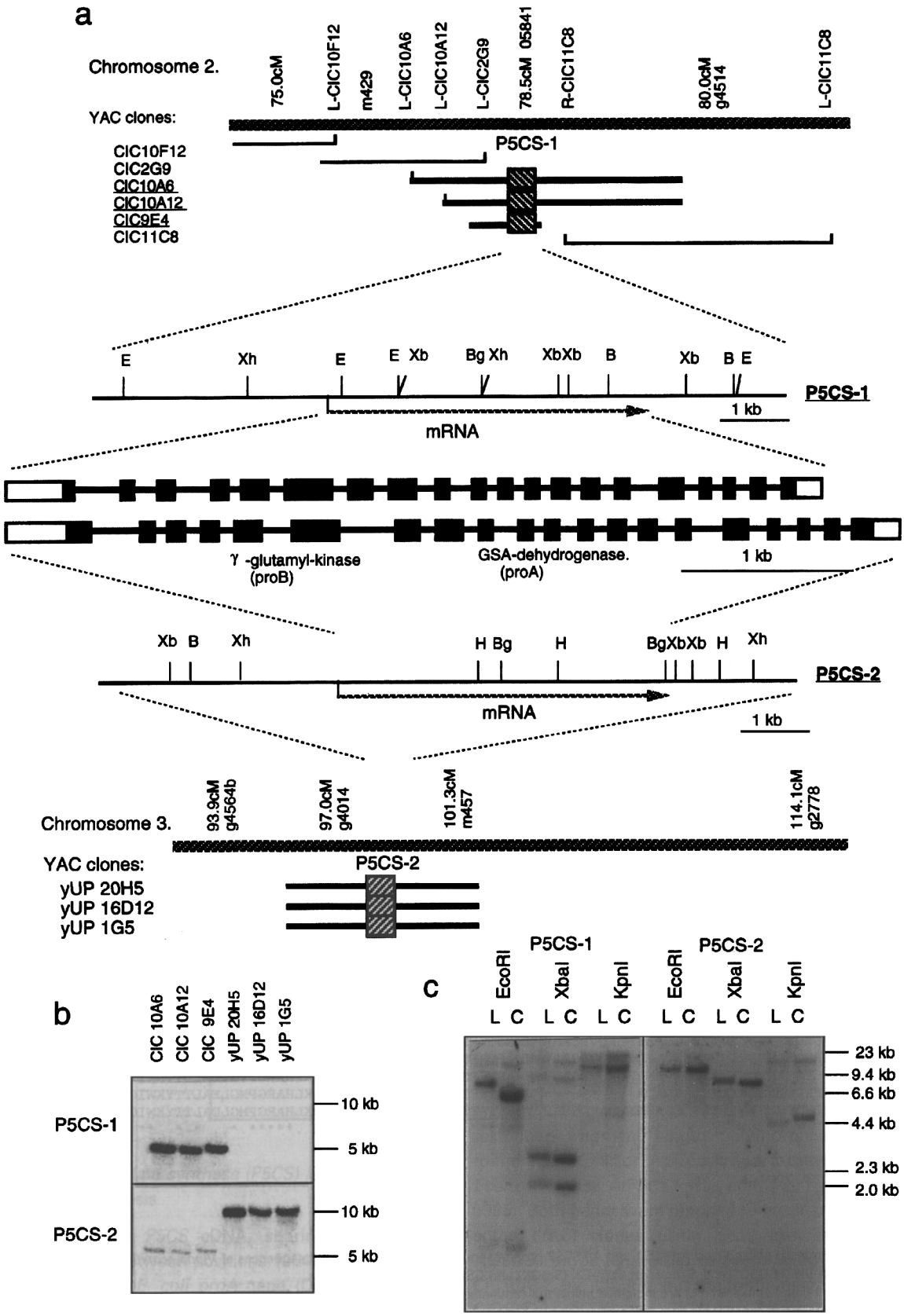


Figure 1. Multiple alignment of predicted AtP5CS1 and AtP5CS2 protein sequences with homologous sequences of *Vigna aconitifolia* P5CS (VaP5CS), *E. coli* γ-GK (EcGK) and GSA-DH (ECGSD), and *S. marcescens* γ-GK (SmGK) and GSA-DH (SMGSD) enzymes.
Amino acid identities (*) and similarities (.) are indicated below the sequence comparison. Sequences in frames show conserved γ-GK and GSA-DH domains, including the putative ATP and NADPH binding domains, leucine-rich regions, as well as amino acids involved in proline inhibition of γ-GK and P5CS enzymes.


a
Chromosome 2.
75.0cM
L-ClC10F12
m429
L-ClC10A6
L-ClC10A12
L-ClC2G9
78.5cM 05841
R-ClC11C8
80.0cM g4514
L-ClC11C8
YAC clones:
ClC10F12
ClC2G9
ClC10A6
ClC10A12
ClC9E4
ClC11C8
P5CS-1
E Xh E E Xb Bg Xh XbXb B Xb B E
P5CS-1
1 kb
mRNA
γ -glutamyl-kinase (proB)
GSA-dehydrogenase. (proA)
1 kb
Xb B Xh H Bg H BgXbXb H Xh
P5CS-2
mRNA
1 kb
Chromosome 3.
93.9cM g4564b
97.0cM g4014
101.3cM m457
114.1cM g2778
YAC clones:
yUP 20H5
yUP 16D12
yUP 1G5
P5CS-2
b
ClC 10A6
ClC 10A12
ClC 9E4
yUP 20H5
yUP 16D12
yUP 1G5
P5CS-1
P5CS-2
10 kb
5 kb
10 kb
5 kb
c
P5CS-1
P5CS-2
EcoRI
XbaI
KpnI
EcoRI
XbaI
KpnI
L C L C L C L C L C L C
23 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

et al., 1995) indicating that *AtP5CS1* is located at position 78.5 cM of chromosome 2, in close proximity of the RFLP marker 05841 between the left end of CIC2G9 and the right end of CIC11C8 YAC clones. The *AtP5CS2* specific probe hybridized to different YACs: yUP20H5, yUP16D12, and yUP1G5 (Figure 2a and b), the chromosomal location of which was unknown. Therefore, the position of the *AtP5CS2* gene and corresponding YAC-contig was mapped by linkage analysis, using recombinant inbreds (Lister and Dean, 1993) and the *Kpn*I RFLP. The *AtP5CS2* gene was located close to position 101.3 cM of chromosome 3 marked by the RFLP marker m457 (Figure 2a).

Characterization of four genomic clones isolated with the *AtP5CS2* probe resulted in a physical map clearly different from that of the *AtP5CS1* locus (Figure 2a; Savouré *et al.*, 1995). Nucleotide sequencing showed that the transcribed region of the *AtP5CS2* gene (EMBL/Genbank X86778) is 4.9 kb, including 20 exons (Figure 2a). Comparison of the exon–intron structure of both *AtP5CS* genes indicated that the lengths of their exons were identical, except for exon 20. The identity between exon sequences of the *AtP5CS* genes ranged from 80% to 94%, whereas the intron sequences showed less conservation (from 39% to 65% identity) and a variation in length. The conserved 5′-splicing sites corresponded to AG/GT at 15 *AtP5CS1* and 16 *AtP5CS2* exon/intron junctions, whereas TG/GT occured at 4 splicing sites in *AtP5CS1* and 3 sites in *AtP5CS2*.

Accumulation of AtP5CS *steady-state mRNAs is tissue specifically regulated by growth factors and stress stimuli*

Northern hybridization with gene specific probes detected significant levels of *AtP5CS1* mRNA in roots, stems, leaves and flowers (Figure 3), as was observed earlier in *Arabidopsis* seedlings using the *AtP5CS1* cDNA probe (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995). In comparison, the levels of *AtP5CS2* steady-state mRNA were about three- to fivefold lower in most plant organs. However, in actively dividing callus and cell suspension cultures the amount of *AtP5CS1* transcript was below the detection limit, whereas the *AtP5CS2* mRNA represented an abundant transcript (Figure 3).

Rapid accumulation of steady-state *AtP5CS* mRNA had been observed in *Arabidopsis* seedlings exposed to drought, NaCl and ABA treatments (Yoshiba *et al.*, 1995). To assess the contribution of *AtP5CS1* and *AtP5CS2* genes to mRNA accumulation induced by environmental and hormonal stimuli, the amount of *AtP5CS* mRNAs was monitored by gene specific probes in seedlings, plant organs and cell cultures. Exposure of light adapted plants to darkness caused a reduction of only 40–50% for the *AtP5CS1* mRNA levels, but did not affect the amount of *AtP5CS2* mRNA. Dehydration of plants resulted in five- to sixfold increase of *AtP5CS1* transcript levels, but triggered only moderate (twofold) accumulation of *AtP5CS2* mRNA (Figure 4a). Exposure of plants to as little as 25 mM NaCl increased both *AtP5CS* transcript levels (as shown for the *AtP5CS1* gene in Figure 4c). In roots treated by NaCl the accumulation of *AtP5CS1* mRNA reached a plateau after 6 h, whereas a slow increase in the amount of *AtP5CS2* mRNA approached a maximum about 24 h after the induction (Figure 4b).

The level of *AtP5CS1* mRNA was increased by ABA treatment to about five- to sixfold in leaves and roots (Figure 5). In comparison to ABA, auxin (2,4-D) resulted in less pronounced (max. threefold) induction of *AtP5CS1* mRNA accumulation in leaves and roots. The results obtained with cytokinin (BAP) treatment of seedlings were more ambiguous. Cytokinin did not affect the accumulation of *AtP5CS1* mRNA in roots, but caused some reduction of *AtP5CS1* mRNA levels in leaves. The effect of hormones

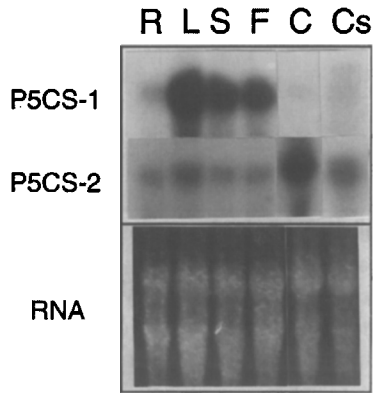


Figure 3. Northern RNA hybridization analysis of *AtP5CS* steady-state mRNA levels in different plant organs and tissues.
20 μg total RNA samples from roots (R), leaves (L), stems (S), flowers (F), root-derived callus (C) and cell suspension cultures (CS) were subjected to Northern RNA blot hybridization with *AtP5CS1* (P5CS-1) and *AtP5CS2* (P5CS-2) gene specific probes.

Figure 2. Genetic and physical mapping of *AtP5CS* genes.
(a) Chromosomal position, physical map, and schematic structure of *AtP5CS1* and *AtP5CS2* genes. Top: Map position of the *AtP5CS1* gene on chromosome 2 defined by an overlapping contig of CIC YAC clones. Middle: Physical map and schematic presentation of exon–intron structure of *AtP5CS1* (Savouré *et al.*, 1995) and *AtP5CS2* genes. Restriction endonuclease cleavage sites are *Eco*RI (E), *Xho*I (Xh), *Xba*I (Xb), *Bam*HI (B), *Bgl*I (Bg), and *Hin*dIII (H). Bottom: Map position of the *AtP5CS2* gene and corresponding yUP YAC clones on chromosome 3. (b) Southern DNA hybridization of YAC clones with *AtP5CS1* and *AtP5CS2* gene specific probes. Slight hybridization signal with CIC YACs is owing to homology between PCR primers used for generation of *AtP5CS2* gene specific probe (see Experimental procedures) and the CIC vector pYAC4 (Creusot *et al.*, 1995). (c) Southern hybridization of *Eco*RI, *Xba*I, and *Kpn*I digested genomic DNAs prepared from *Arabidopsis* seedlings of Col-1 (C) and La-er (L) ecotypes with *AtP5CS1* and *AtP5CS2* probes. Restriction fragment length polymorphism was detected with the *AtP5CS1* probe by *Eco*RI, and the *AtP5CS2* probe by *Kpn*I.

on the regulation of *AtP5CS2* transcript levels was considerably different. The induction of *AtP5CS2* mRNA accumulation by ABA was slower than that of *AtP5CS1* mRNA in leaves, and ABA-treatment did not increase the levels of *AtP5CS2* mRNA in roots. In leaves both auxin and cytokinin treatments resulted in elevated *AtP5CS2* transcript levels 24 h after the hormone application, but in roots neither auxin nor cytokinin appeared to affect the accumulation of *AtP5CS2* mRNA significantly.

In dividing cell cultures an increase of both *AtP5CS1* and *AtP5CS2* transcript levels was detected very early after ABA addition (Figure 5). The levels of *AtP5CS1* and *AtP5CS2* mRNAs reached a maximum, respectively, within 1 h and 20 min after the ABA treatment. However, the amounts of both *AtP5CS* mRNAs started to decay about 6 h later, and reached basic levels 48 h after the ABA addition. In comparison, the induction of the apparently silent *AtP5CS1* gene in NaCl-treated cell cultures was detectable after 1 h, but reached a maximum only 6 h after exposing the cells to NaCl. NaCl-induced accumulation of the *AtP5CS2* mRNA, starting from a higher basic level, also showed a slight increase after 1 h, and reached a maximum 6 h later (Figure 4b). In contrast to the transient accumulation of *AtP5CS* mRNAs in ABA-treated cells, the induced levels of both *AtP5CS* mRNAs were still observed 48 h after treating the cells with NaCl. The data thus suggested that, if ABA was implicated in NaCl-induction, a lack of initial fast response of *AtP5CS* mRNA accumulation to NaCl could reflect either a limited supply of ABA in cell suspension cultures, or a need for protein synthesis preceding the induction of *AtP5CS* mRNA accumulation by NaCl, or both.

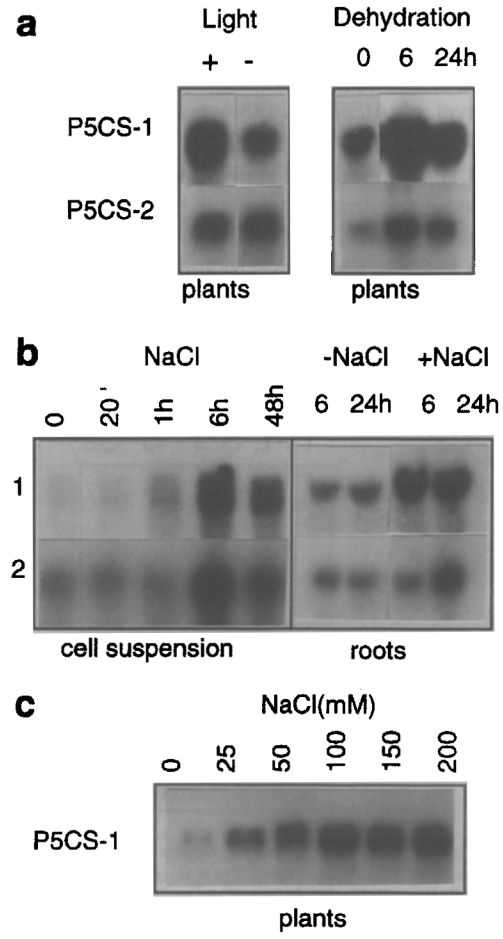


Figure 4. Regulation of the level of steady-state *AtP5CS* mRNAs by environmental stimuli.
(a) Four weeks old *Arabidopsis* seedlings grown in SG-medium were transferred for 3 days from light to dark (left) or exposed to dehydration for 6 and 24 h (right) before RNA hybridization with *AtP5CS* gene specific probes P5CS-1 and P5CS-2. (b) The effect of salt-stress on the accumulation of *AtP5CS* mRNAs in suspension culture of dividing cells (left) and roots (right). The cell suspension culture and roots were treated by 200 mM NaCl and samples were collected at different time intervals as indicated above the RNA blots obtained with the *AtP5CS* gene specific probes. (c) RNA was purified from *Arabidopsis* seedlings grown for 8 days on SG-medium containing different concentrations of NaCl indicated above the Northern blot, and hybridized with the *AtP5CS1* probe.

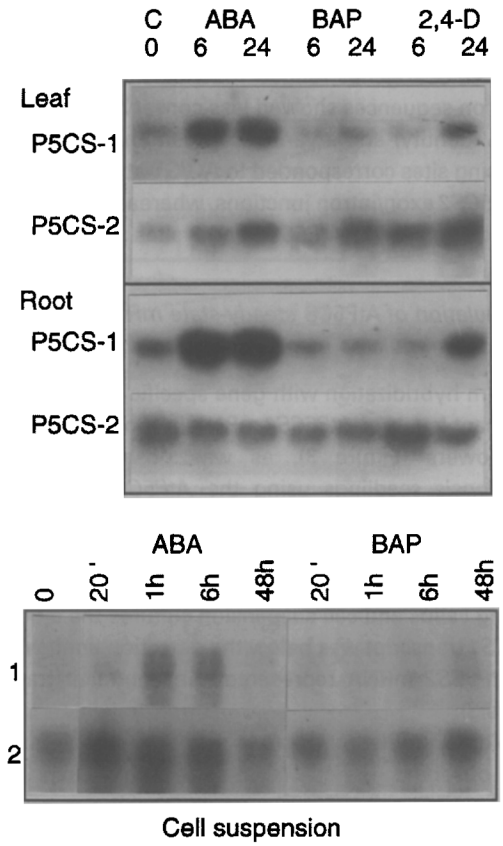
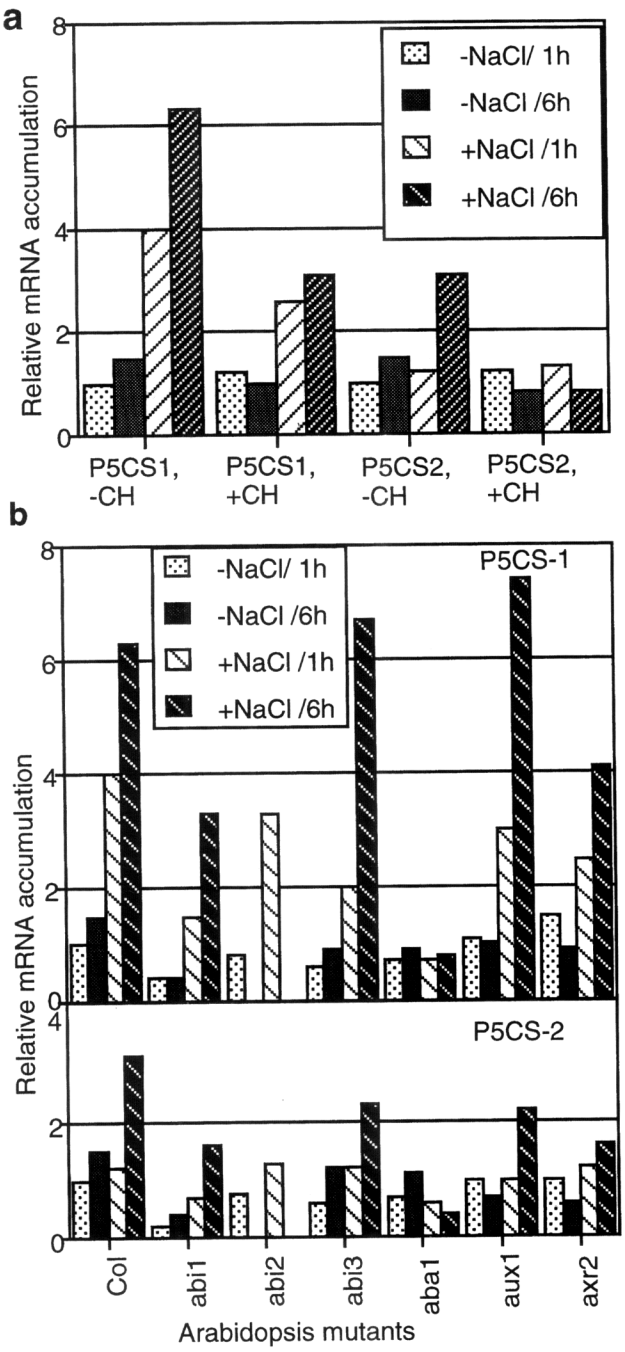


Figure 5. The effect of plant growth factors on the regulation of *AtP5CS* mRNA accumulation in leaves, roots, and suspension culture of dividing cells.
Four-week-old seedlings grown in SG-medium were treated with 1 μM ABA, or 1 mg l^{-1} BAP, or 1 mg l^{-1} 2,4-D for 6 or 24 h (top). Cultured dividing cells were washed with hormone-free medium and subcultured in MSAR-medium containing 1 μM ABA or 1 mg l^{-1} BAP (bottom). Plant samples were collected at different time intervals shown above the autoradiographs, and used for Northern RNA hybridizations with *AtP5CS* gene specific probes P5CS-1 and P5CS-2.

NaCl-induced P5CS *mRNA accumulation in Arabidopsis seedlings: involvement ABA signalling and protein synthesis*

In order to correlate our data with previous reports (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995), we performed a quantitative analysis of *AtP5CS* mRNA accumulation in *Arabidopsis* seedlings. Clearly, this approach could not be used to observe tissue specific differences in *AtP5CS* gene regulation as described above, but reflected changes in the total amount of *P5CS* mRNAs in whole plants. The amounts of *AtP5CS1* and *AtP5CS2* mRNAs were measured by phosphorimage analysis of Northern blots, to determine whether the inhibition of protein synthesis by cycloheximide, or gene mutations affecting either ABA perception or biosynthesis, would alter the kinetics of NaCl-induced *AtP5CS* transcript accumulation.



A pretreatment of seedlings with cycloheximide caused only a minor reduction (25%) in the accumulation of *AtP5CS1* mRNA during the first hour of subsequent NaCl induction (Figure 6a). However, cycloheximide completely prevented a further increase of transcript levels, which reached a maximum 6 h after NaCl addition in seedlings untreated with cycloheximide. The levels of *AtP5CS2* mRNA increased more slowly and reached a maximum, corresponding to two- to threefold of basal levels, within 6 h. As observed for *AtP5CS1*, the slow induction of *AtP5CS2* mRNA accumulation was also completely abolished by cycloheximide pretreatment of seedlings (Figure 6a). The data thus indicated that protein synthesis is probably required for continuous accumulation and maintenance of induced levels of *AtP5CS* mRNAs during salt stress.

NaCl-induced accumulation of *AtP5CS* mRNAs was also monitored in mutant *Arabidopsis* seedlings, including the ABA deficient *aba1-1* (Koornneef *et al.*, 1982), the ABA-insensitive *abi1-1, abi2-1* and *abi3-1* (Koornneef *et al.*, 1984), the auxin/ethylene resistant *aux1–7* (Maher and Martindale, 1980; Pickett *et al.*, 1990), and the auxin/ethylene/ABA resistant *axr2* (Wilson *et al.*, 1990) mutants (Figure 6b). In the ABA deficient *aba1-1* mutant, NaCl treatment failed to induce any increase of basic *AtP5CS* transcript levels, demonstrating that ABA is absolutely essential as signalling molecule for salt-induced activation of both *AtP5CS1* and *AtP5CS2* genes. The *abi2-1, abi3-1* and *aux1-7* mutations did not affect the peak levels of *AtP5CS1* and *AtP5CS2* transcripts, which were comparable in these mutants and the wild-type control after 6 h of NaCl induction. In contrast, both *abi1-1* and *axr2* mutations reduced the *AtP5CS1* transcript levels to about half of the values

Figure 6.
(a) Comparison of the levels of *AtP5CS* mRNAs in cycloheximide treated wild-type *Arabidopsis* seedlings. Two weeks old plantlets were treated in either liquid SG-medium (-NaCl) or SG-medium containing 200 mM NaCl (+NaCl) for 1 and 6 h, following incubation with 0.2 mM cycloheximide for 1 h. RNA samples (20 μg) prepared after the treatments were hybridized with *AtP5CS* gene specific probes. The hybridization signals were quantitatively measured by phosphorimage analysis, and plotted to show relative accumulation of *AtP5CS1* (P5CS1) and *AtP5CS2* (P5CS2) mRNAs.
(b) Wild-type (Col-1), as well as *abi1-1, abi2-1, abi3-1, aba1-1, aux1-7,* and *axr2* mutant *Arabidopsis* seedlings were cultured for 1 or 6 h in either SG-medium (-NaCl) or SG-medium containing 200 mM NaCl. Relative levels of *AtP5CS* mRNAs were determined by RNA hybridization and phosphorimage analysis as described above. The *AtP5CS* RNA levels were determined only after 1 h of similar treatments in *abi2-1* seedlings. Each column diagram represents the mean of two independent measurements, implying a standard deviation of mean values between 0.1 and 0.175.

detected in wild-type control seedlings following 1 and 6 h of salt-induction. In comparison with wild-type a general decrease of basic *AtP5CS1* mRNA levels was observed in the *abi1-1* mutant, also in the absence of salt induction. In contrast, the *axr2* mutation did not affect the basic *AtP5CS1* mRNA levels, but reduced the induction of *AtP5CS1* mRNA accumulation during salt treatment. A similar effect of *abi1–1* and *axr2* mutations on the accumulation of *AtP5CS2* mRNA was detected only after 6 h, owing to a slower increase of transcript levels. These data thus showed that 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*.

Discussion

Genes involved in glutamate-derived proline biosynthesis

The identification of rate-limiting steps controlling the accumulation of proline during salt and water stress in plants has been a key issue of earlier studies (Hanson and Hitz, 1992; Delauney and Verma, 1993). cDNAs coding for the Δ^1-pyrroline-5-carboxylate reductase (P5CR) were cloned from soybean by functional complementation of the *E.coli proC* mutation (Delauney and Verma, 1990), and subsequently from pea and *Arabidopsis* (Williamson and Scolum, 1992; Verbruggen *et al.*, 1993). Because the induction of *AtP5CR* gene expression was detectable only 24 h after salt-stress in *Arabidopsis*, and not at all in response to dehydration and exogeneous ABA (Yoshiba *et al.*, 1995), P5CR is not thought to play a regulatory role in the stress-induced accumulation of proline. By functional complementation of the *E.coli proB* mutation, a *Vigna* cDNA was subsequently isolated and found to encode a fusion protein consisting of γ-glutamyl kinase and γ-glutamyl phosphate reductase enzyme domains (Hu *et al.*, 1992). The γ-glutamyl kinase (γ-GK) activity of this bifunctional Δ^1-pyrroline-5-carboxylate synthase (P5CS) enzyme was shown to be 30 times less sensitive to feed-back inhibition by proline than the *E. coli* γ-GK enzyme. Accordingly, amino acid residues involved in proline inhibition of bacterial γ-GK enzymes (Csonka *et al.*, 1988; Omori *et al.*, 1992) were not found to be conserved in the *Vigna* P5CS sequence (Hu *et al.*, 1992).

Nonetheless, the bacterial model, identifying the inhibition of γ-GK by proline as rate limiting step in proline biosynthesis (Csonka and Hanson, 1991), was applied for plants because mutational analysis of the *Vigna* P5CS identified novel amino acid positions affecting the inhibition of P5CS enzyme by proline (Zhang *et al.*, 1995). These amino acid residues were found to be conserved in an *Arabidopsis* P5CS protein, the sequence of which was deduced from genomic and cDNA clones (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995). Because δ-transamination of ornithine to P5C occurs preferentially under normal conditions, whereas the conversion of glutamate to P5C is dominant under stress (Rhodes *et al.*, 1986), the expression and activity of P5CS was proposed to directly control the accumulation of proline in response to salinity and dehydration (Delauney and Verma, 1993). In fact, studies of the *P5CS* expression in *Arabidopsis* seedlings demonstrated that proline accumulation is preceded by a rapid increase of *P5CS* mRNA levels during exposure to dehydration, exogeneous ABA, and salinity (Yoshiba *et al.*, 1995).

Because independent reports suggested that P5CS is encoded by a single gene in *Arabidopsis* (Savouré *et al.*, 1995; Yoshiba *et al.*, 1995), it seemed that the question whether proline accumulation is necessary and sufficient to confer stress tolerance to plants may be answered by isolation of a *P5CS* gene mutation. Before initiating a search for insertional mutations in *P5CS*, we performed a rigorous screening of *Arabidopsis* cDNA and genomic DNA libraries, using a domain of *Vigna P5CS* cDNA as probe. Unexpectedly, two classes of cDNA clones were identified, one present only in seedling-derived cDNA libraries, and another in a cDNA library made from suspension cultures of dividing cells. The *AtP5CS* cDNAs shared a sequence identity of 82%, but their 3′ and 5′ untranslated sequences showed only 53 and 54% identity. To confirm that indeed more than one *AtP5CS* gene is present in *Arabidopsis*, several genomic clones were isolated and characterized. In addition, Southern DNA hybridization analysis using Columbia and Landsberg ecotypes demonstrated that *Arabidopsis* contains two different *P5CS* genes, *AtP5CS1* and *AtP5CS2*, which were mapped respectively to chromosome 2-78.5 and close to position 101.3 of chromosome 3 by RFLP linkage analysis and YAC hybridization. Nucleotide sequence analysis of genomic and cDNA clones indicated that the lengths of 19 out of 20 exons are identical in both *AtP5CS* genes, whereas their intron sequences are less conserved and vary in length.

Redundancy of genes involved in amino acid biosynthesis pathways is common in plants. Recently, a similar study indicated that two *P5CS* genes are also present in alfalfa (Zilberstein and Szabados, unpublished). Divergence between members of gene families during evolution appear to often begin with changes in transcriptional regulation, which may be followed by alterations in their biochemical function (for review see Pickett and Meeks-Wagner, 1995). A repeated analysis of gene expression was therefore necessary to determine the contribution of *AtP5CS1* and *AtP5CS2* genes to the accumulation of *P5CS* mRNA, preceding the raise of proline production during salt or water stress (Yoshiba *et al.*, 1995).

Differential regulation of AtP5CS *genes*

RNA hybridization analyses indicated that about 60 to 80% of steady-state *P5CS* RNA results from transcription of the *AtP5CS1* gene, although significant amounts of steady-state *AtP5CS2* transcript were also detected in plant organs. Nonetheless, no cDNA clone corresponding to polyadenylated *AtP5CS2* mRNA was found in cDNA libraries made from plants, suggesting that yet unknown tissue specific differences between processing or stability of *AtP5CS* mRNAs may exist. In contrast, cDNA libraries prepared from suspension cultures of dividing cells did not contain *AtP5CS1* cDNA. Moreover, RNA hybridization data showed that *AtP5CS2* mRNA is abundant, whereas *AtP5CS1* transcript is not detectable in cell suspensions.

As observed earlier (Yoshiba *et al.*, 1995), dehydration, ABA and NaCl treatments resulted in a rapid induction of *AtP5CS* mRNA accumulation in *Arabidopsis* seedlings. However, the accumulation of *AtP5CS* mRNAs monitored by gene specific probes revealed characteristic tissue specific differences in response to treatments by salt and plant growth factors, including ABA and auxin. The most striking difference between *AtP5CS1* and *AtP5CS2* mRNA levels was detected in cell suspension cultures. In dividing cells the *AtP5CS1* mRNA was barely detectable in the absence of salt or ABA treatment, whereas *AtP5CS2* was found to be expressed as a constitutive 'house-keeping' gene. Following induction by salt or ABA, the level of *AtP5CS2* mRNA increased to two- to threefold, whereas the amount of *AtP5CS1* mRNA reached about 10 to 15% of *AtP5CS2* mRNA in dividing cell cultures. Peak values of *AtP5CS* mRNA levels were reached after 1 h in cell suspensions treated by ABA, but started to decline 6 h later, and dropped to basic levels 48 h after the ABA treatment. In salt-treated cells maximal values of *AtP5CS* mRNA accumulation were observed only after 6 h, but the induced transcript levels were maintained even 48 h after the salt-treatment. This suggested that an early response mediated by ABA may be required for salt-induction of *AtP5CS* transcription.

Accumulation of AtP5CS *mRNAs is ABA-dependent during salt-stress*

Thus far only limited information is available about signalling pathways controlling plant gene expression in response to drought and salinity (Chandler and Robertson, 1994; Ingram and Bartels, 1996). The analysis of certain ABA-responsive genes, such as *Rab16A*, suggests that ABA-induction involves an immediate early transcriptional response, occuring in the presence of protein synthesis inhibitors (Mundy and Chua, 1988). However, ABA-induction of other genes is shown to be inhibited by cycloheximide, indicating a requirement for protein synthesis (Nakagawa *et al.*, 1996). Proline accumulation induced by ABA or NaCl in barley and *Arabidopsis* was also found to be inhibited by cycloheximide (Stewart *et al.*, 1986; Verbruggen *et al.*, 1993), but it is unknown whether this resulted from the inhibition of synthesis or translation of *P5CR* and/or *P5CS* mRNAs.

The induction of *AtP5CS* mRNA synthesis in salt-treated *Arabidopsis* seedlings follows roughly an exponential curve (Yoshiba *et al.*, 1995). Our data show that the fast linear phase of induction, which contributes to about five- to sixfold increase of mRNA levels within 1 h, is not inhibited by cycloheximide, thus probably represents an immediate early response. In salt-treated seedlings this early response is primarily attributed to activation of the *AtP5CS1* gene (yielding about 60–80% of total *AtP5CS* mRNA), whereas the *AtP5CS2* mRNA level shows only a marginal increase during this early phase. (However, owing to high basic levels of *AtP5CS2* mRNA, the activation of the apparently silent *AtP5CS1* gene does not lead to a significant increase of total *AtP5CS* mRNA levels during this early phase in salt-treated dividing cells.) The total amount of *AtP5CS* mRNA reaches a maximum 6 h after the salt-treatment. This second slow phase of salt-induction is abolished by cycloheximide. The inhibition of protein synthesis by cycloheximide prevents the increase of *AtP5CS1* mRNA levels following the early phase, and completely inhibits the accumulation of *AtP5CS2* mRNA in *Arabidopsis* seedlings. Because the early phase of *AtP5CS1* mRNA accumulation is inhibited in the ABA-deficient *aba1* mutant, it is probable that salt-induced activation of the *AtP5CS1* gene is controlled by an ABA-mediated early transcriptional response. The fact that *AtP5CS2* mRNA accumulation is also undetectable in the *aba1* mutant suggests, on the contrary, that ABA-signalling is also implicated in the slow, cycloheximide sensitive phase of salt-induction.

Signalling functions modulating the salt-induced accumulation of AtP5CS *mRNAs*

Perception of ABA in *Arabidopsis* is abolished by several gene mutations, including *abi1-1*, *abi2-1*, and *abi3-1*, which confer ABA-insensitivity. Our observation that the *abi* mutations differently affect the accumulation of *AtP5CS* mRNAs supports the model proposing that the *abi* mutations define at least two different signalling pathways (Finkelstein and Zeevaart, 1994; Gosti *et al.*, 1995; Koornneef *et al.*, 1984; Parcy *et al.*, 1994). Our data show that salt-induced accumulation of both *AtP5CS* mRNAs is reduced, but not completely inhibited, by the *abi1-1* mutation, and unaffected in the *abi2-1* and *abi3-1* mutants. This data correlates with the observation that ABA-induced accumulation of proline is reduced in *abi1-1* seedlings, but not affected by the *abi3-1* mutation (Finkelstein and

Somerville, 1990). An analogous influence of *abi1-1* and *abi2-1* mutations on the expression of ABA-responsive *cor6.6* gene was observed by Patel *et al.* (1994). Other studies also indicate that *ABI1* is required for the expression of most ABA-regulated genes, whereas the yet unknown *ABI2* gene affects the regulation of a smaller subset of ABA-induced genes in vegetative tissues (Finkelstein and Somerville, 1990; Patel *et al.*, 1994). Because *ABI1* codes for a Ca^{2+}-modulated protein phosphatase 2C (Leung *et al.*, 1994; Meyer *et al.*, 1994), it is likely that protein phosphorylation plays an important role during the early phase of salt-induced *AtP5CS* mRNA accumulation. On the contrary, our data also show that the seed-specific *ABI3* gene, encoding a homolog of maize VP-1 protein (Giraudat *et al.*, 1992), has no effect on the expression of *AtP5CS* genes in *Arabidopsis* seedlings.

Because tissue specific induction of *AtP5CS* mRNA accumulation is also modulated by auxin, we examined the expression of *AtP5CS* genes in the auxin resistant mutants *aux1-7* (Maher and Martindale, 1980) and *axr2* (Estelle and Klee, 1994). The *aux1-7* mutation, which confers resistance to auxin and ethylen (Pickett *et al.*, 1990), did not modify the induction of *AtP5CS* genes during salt-stress. In contrast, the *axr2* gene mutation, which results in resistance to auxin, ethylene and ABA, caused a reduction in the *AtP5CS* mRNA accumulation during salt-stress, whereas the *abi1-1* mutation resulted in a reduction of basic *AtP5CS* mRNA levels even in the absence of salt treatment. Thus, *axr2* appears to perform a broader signalling function which may establish a link between auxin and ABA signal transduction. In any case, *axr2* and *abi1* appear to act in a common ABA signalling pathway which controls both basic level and salt-induced accumulation of *AtP5CS* mRNAs in *Arabidopsis* seedlings.

Experimental procedures

Plant growth conditions

Following germination, *Arabidopsis thaliana* (Col-1) seedlings were grown in glass jars on seed-germination (SG) medium (Koncz *et al.*, 1994) at 22°C using 16 h light and 8 h dark cycle. Callus cultures were initiated from roots and maintained on solid MSAR1 medium (Koncz *et al.*, 1994). Root-derived cell suspension cultures were established and subcultured weekly in MSAR-medium containing 1 mg l^{-1} 2,4-D (2,4-dichloro-phenoxyacetic acid) as described (Mathur *et al.*, 1995).

Seedlings 2 or 4 weeks old were subjected to stress or hormonal treatments by transferring them into 250 ml Erlenmeyer flasks containing 30 ml liquid SG-medium. For hormonal treatments 1 μM ABA (abscisic acid), or 1 mg l^{-1} 2,4-D, or 2 mg l^{-1} BAP (6-benzyl-aminopurine) was added to the SG liquid medium. Conditions for salt-stress were assayed as shown in Figure 4(c), whereas in other experiments 200 mM NaCl was used for salt-induction of 2 weeks old wild-type (Col-1) and mutant (*aba1-1*, *abi1-1*, *abi2-1*, *abi3-1*, *aux1-7* and *axr2*) seedlings (Koornneef *et al.*, 1982, 1984; Maher and Martindale, 1980; Wilson *et al.*, 1990). To block protein synthesis, 0.2 mM cycloheximide was added to the medium 1 h before the salt-induction. Desiccation of seedlings grown in tissue culture was induced by removing the closure of culture vessels and maintaining the plants in open jars in the growth chamber. Plant materials were harvested in liquid nitrogen for RNA preparation.

Characterization of AtP5CS *genomic and cDNA clones*

Arabidopsis cDNA libraries made in λ-ZAPII from cell suspension culture (kindly donated by I. Somssich, MPI, Cologne) and in λgt10 from seedlings (kindly donated by A. Bachmair, University Vienna) were screened by an oligonucleotide probe containing a segment of *Vigna P5CS* cDNA between positions 1954 and 2037 (Hu *et al.*, 1992) as described (Sambrook *et al.*, 1989). The cDNA clones were characterized by physical mapping and nucleotide sequencing, then used for testing the complementation of *E. coli* proline auxothropic mutant strains JM83 (F⁻, *ara*, Δ(*lac-proAB*), *rpsL*, [*θ*80d, *lac*Δ, (*lacZ*)M15]), CSH26 (Δ(*lac-proAB-gpt*), *thi*, *ara*), HB101 (F⁻, Δ(*gpt-proAB*), *leuB*, *supE*, *ara*, *galK*, *lacY*, Δ(*mcrC-mrr*), *rpsL*, *xyl*, *mtl*, *recA*), G9 (F⁻, *proA*, *leu*, *thr*, *thi*, *lac*, *rpsL*) and G13 (F⁻, *proB*, *leu*, *thr*, *thi*, *lac*, *rpsL*). The complementation assays were performed by transformation of proline auxotropic *E. coli* mutants with plasmid pC8–2 that contained the complete coding sequence **of *AtP5CS1* cDNA (between positions 1 and 2322, EMBL/Genbank X86777) in sense orientation under control of the *lacZ* promoter** in pBluescript (Genbank X52328). Plasmid pC8-2 complemented the *proAB* deletions in strains JM83, CSH26, and HB101, as well as the *proA* and *proB* mutations in strains G9 and G13, respectively. In contrast, no complementation of these mutations was observed with plasmid pC8-1 that carried the same cDNA insert in reverse orientation in pBluescript. In plasmid pQB3, the sequence of *AtP5CS2* cDNA (between positions 903 and 2440, EMBL/Genbank Y09355) was cloned in the *Kpn*I-site of pQE30 vector (Qiagen), thus it was fused in frame to a methionine codon followed by codons for 6xHis-tag. The GSA-DH domain of *AtP5CS2* coding region complemented only the *proA* mutation in *E. coli* strain G9.

The 5′-region of *AtP5CS2* cDNA was cloned by RACE-PCR amplification (Frohman *et al.*, 1988), using a vector specific primer of 25 bp (5′-CTCGAAATTAACCCTCACTAA AGGG-3′) derived from the T3 promoter region of pBluescript SK(+), and a *AtP5CS2* specific primer (5′-CACGAGAAGTAGTATCTACGAC CGG-3′), corresponding to the 5′-end of longest available cDNA clone. The PCR amplification was performed using 35 cycles of 95°C for 30 s, 65°C for 1 min and 72°C for 1 min, followed by final extension at 72°C for 5 min. The amplified DNA was digested by *Kpn*I and *Bam*HI, gel purified and cloned into corresponding sites of pBluescript. Nucleotide sequence of 5 clones was determined, then a full-length *AtP5CS2* cDNA was reconstructed using the unique *Kpn*I site in the PCR product and partial cDNA clone.

To isolate the *AtP5CS2* gene, 300 000 plaques from an *Arabidopsis* genomic DNA library made in λ-GEM11 vector (obtained from the DFG *Arabidopsis* DNA Stock Center, Cologne) were screened using the 3′-end of *AtP5CS2* cDNA as probe. Four genomic clones were characterized by physical mapping using Southern DNA hybridization with the *AtP5CS2* cDNA probe, then DNA fragments carrying the gene were subcloned and sequenced, using a Pharmacia sequencing kit. Sequence analyses were performed using a GCG programme package adapted to UNIX.

Chromosomal mapping of AtP5CS *genes*

For Southern DNA hybridization analysis DNA was prepared from *Arabidopsis* seedlings of Columbia (Col-1) and Landsberg (La-er)

ecotypes (Dellaporta *et al.*, 1983) and hybridized with either *AtP5CS1* or *AtP5CS2* cDNA probes at 65°C overnight as described (Sambrook *et al.*, 1989). Restriction fragment length polymorphism (RFLP) detected by *Kpn*I was used as marker for genetic mapping of the *AtP5CS2* gene using recombinant inbred lines (Lister and Dean, 1993). CIC and yUP yeast artificial chromosome (YAC) libraries (Creusot *et al.*, 1995; Ecker *et al.*, 1990) were screened using *AtP5CS* gene specific probes (see below). In order to confirm the mapping data, DNA from the YAC clones was isolated and subjected to Southern hybridization as described (Matallana *et al.*, 1992).

Determination of AtP5CS *transcript levels*

RNAs were extracted from plant organs, calli and cell cultures according to Pawlowski *et al.* (1994). RNA concentrations were determined spectrophotometrically (Sambrook *et al.*, 1989), then equal amounts of RNA samples (15 or 20 μg) were size separated in denaturing agarose gels, visualized by ethidium bromide staining, blotted to Hybond N^+-filters, and hybridized with radioactively labelled probes (Sambrook *et al.*, 1989). Following hybridization for 24 h at 42°C in a buffer containing 50% formamide (Sambrook *et al.*, 1989), the filters were washed twice with 3 × SSC, 0.1% SDS and twice with 0.1 × SSC, 0.1% SDS at 60°C. Gene specific probes were prepared by PCR amplification of the divergent 3'-ends of *AtP5CS1* and *AtP5CS2* cDNAs. 35 cycles of PCR amplification were performed at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min followed by final extension at 72°C for 5 min, using a T7-promoter primer for pBluescript (5'-TGTAATACG ACTCACTATAGGGCG-3') and another primer (5'-GTTTACACCCATA/CAGGA-3') annealing to the *AtP5CS* cDNAs. The Northern blots were standardized using an ubiquitin probe as described (Szekeres *et al.*, 1996). Quantitative analysis of Northern hybridization signals was performed by PhosphorImager-445SI (Molecular Dynamics) and IMAGE QUANT™ version 4.1 sofware.

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