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 Δ1-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 m47 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 ab1 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 a11 and axr2, affecting ABA-perception in Arabidopsis, reduce the accumulation of both AtP5CS mRNAs during salt-stress, whereas ABA-signalling functions defined by the a92 and a93 mutations have no effect on salt-induction of the AtP5CS genes.

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; Yoshih 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 (Delaunay 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 (GSSA) in plants by a bifunctional Δ1-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 GSSA via pyrroline-5-carboxylate (P5C) by the Δ1-pyrroline-5-carboxylate reductase (P5CR) enzyme (Delaunay 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., 1988; Soko et al., 1992).

As is the case in bacteria, proline controls the γ-GK activity of P5CS also in plants by feedback 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; Yoshiha 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 (Yoshiha 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; Yoshiha 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; Yoshiha 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 meristematic tissues.

Results

Δ1-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 Yoshiha 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 7 17 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 KpnI for AtP5CS2, and a previously reported EcoRI 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 C10A6, C10A12 and C10E4, but not to the neighbouring clones C10F12, C10G9 and C11C8 (Figure 2a and b). This allowed us to refine the previous mapping data (Savouré
Figure 1. Multiple alignment of predicted AtPSCS1 and AtPSCS2 protein sequences with homologous sequences of Vigna aconitifolia PSCS (VaPSCS), 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 probe inhibition of γ-GK and PSCS enzymes.
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 yUP11G8 (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 KpnI 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 occurred 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., 1996) and AtP5CS2 genes. Restriction endonuclease cleavage sites are EcoRI (E), XhoI (Xh), XbaI (Xb), BamHI (B), BglII (Bg), and HindIII (H). Bottom: Map position of the AtP5CS2 gene and corresponding yUP11 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 EcoRI, XbaI, and KpnI digested genomic DNAs prepared from Arabidopsis seedlings of Col-1 (C) and LER (L) ecotypes with AtP5CS1 and AtP5CS2 probes. Restriction fragment length polymorphism was detected with the AtP5CS1 probe by EcoRI, and the AtP5CS2 probe by KpnI.
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 mRNA 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.

![Diagram](image)

**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.

![Diagram](image)

**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 0 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 (Savoure et al., 1995; Yoshida 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 phosphorimagine 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; Fickett 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 phosphorimagine 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 phosphorimagine 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 AtP6CS2 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 Δ1-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 Solum, 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 δ-glutamyl phosphate reductase enzyme domains (Hu et al., 1992). The γ-glutamyl kinase (γ-GK) activity of this bifunctional Δ1-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 (Savoure 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 (Savoure 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 insertion 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 (Yoshida 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, occurring in the presence of protein synthesis inhibitors (Mundy and Chua, 1998). 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 (Yoshida 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 Ca2+-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 aux2 (Estelle and Klee, 1994). The aux1-7 mutation, which confers resistance to auxin and ethylene (Pickett et al., 1990), did not modify the induction of AtP5CS genes during salt stress. In contrast, the aux2 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, aux2 appears to perform a broader signalling function which may establish a link between auxin and ABA signal transduction. In any case, aux2 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-dichlorophenoxyacetic 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-benzylaminopurine) 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 (abi1-1, abi1-1, abi2-1, abi3-1, aux1-7 and aux2) seedlings (Koomneef 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 a oligonucleotide probe containing a segment of Vigna P5CS cDNA between positions 1954 and 2037 (Hu et al., 1992) as described (Sambrrok et al., 1989). The cDNA clones were characterized by physical mapping and nucleotide sequencing, then used for testing the complementation of E. coli proline auxotrophic mutant strains JM83 (F−, ara, Δlac-proAB), rpsL, Δβ60, lacZ, (lacZ/M15), CSH26 (Δlac-proAB-gpt, thi, ars), HB101 (F−, Δgpt-proAB, leuB, supE, ara, galK, lacY, ΔMcrCmR, 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 auxotrophic E. coli mutants with plasmid pCB-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 pCB-2 complemented the proA deletion 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 pCB-1 that carried the same cDNA insert in reverse orientation in pBluescript. In plasmid pQG3, the sequence of AtP5CS2 cDNA (between positions 902 and 2440, EMBL/Genbank Y09355) was cloned in the KpnI 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′-CTCGAATTACACCTACTA AAGG-3′) derived from the T3 promoter region of pBluescript SK+(+) and a AtP5CS2 specific primer (5′-CAGCAAGAAGTAGTATCAGAC 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 KpnI and BamHI, 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 KpnI 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 (Le-er)
ecotypes (Dellaporta et al., 1983) and hybridized with either AtPSCS1 or AtPSCS2 cDNA probes at 65°C overnight as described (Sambrook et al., 1989). Restriction fragment length polymorphism (RFLP) detected by KpnI was used as marker for genetic mapping of the AtPSCS2 gene using recombinant inbred lines (Lister and Dean, 1993). CIC and yUP yeast artificial chromosome (YAC) libraries (Creusot et al., 1995; Eckert et al., 1990) were screened using AtPSCS 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 (Mataillana et al., 1992).

Determination of AtPSCS 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 Hybrid Bond N 1-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 x SSC, 0.1% SDS and twice with 0.1 x SSC, 0.1% SDS at 60°C. Gene specific probes were prepared by PCR amplification of the divergent 3' ends of AtPSCS1 and AtPSCS2 cDNAs. 35 cycles of PCR amplification were performed at 95°C for 30 s, 56°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 ACT-CACATAGGCGG-3’) and another primer (5’-GTTTACACCAT/ CAGGA-3’) annealing to the AtPSCS 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 Phosphorimeter-444SS (Molecular Dynamics) and Image Quant™ version 4.1 software.

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