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# Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in *Arabidopsis*

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**Abstract**

Osmotic stress-induced accumulation of proline, an important protective osmolyte in higher plants, is dependent on the expression of $\Delta^1$-pyrroline-5-carboxylate synthase (P5CS) and proline dehydrogenase (PDH) enzymes that catalyze the rate-limiting steps of proline biosynthesis and degradation, respectively. Proline metabolism is modulated by differential regulation of organ specific expression of *PDH* and duplicated *P5CS* genes in *Arabidopsis.* Stimulation of proline synthesis by abscisic acid (ABA) and salt stress correlates with a striking activation of *P5CS1* expression. By contrast, *P5CS2* is only weakly induced, whereas *PDH* is inhibited to different extent by ABA and salt stress in shoots and roots of light-grown plants. Proline accumulation and light-dependent induction of *P5CS1* by ABA and salt stress is inhibited in dark-adapted plants. During dark adaptation *P5CS2* is also down-regulated, whereas *PDH* expression is significantly enhanced in shoots. The inhibitory effect of dark adaptation on *P5CS1* is mimicked by the steroid hormone brassinolide. However, brassinolide fails to stimulate *PDH*, and inhibits *P5CS2* only in shoots. Proline accumulation and induction of *P5CS1* transcription are simultaneously enhanced in the ABA-hypersensitive *prl1* and brassinosteroid-deficient *det2* mutants, whereas *P5CS2* shows enhanced induction by ABA and salt only in the *det2* mutant. In comparison, the *prl1* mutation reduces the basal level of *PDH* expression, whereas the *det2* mutation enhances the inhibition of *PDH* by ABA. Regulation of *P5CS1* expression thus appears to play a principal role in controlling proline accumulation stimulated by ABA and salt stress in *Arabidopsis.*

*Abbreviations: det2*, de-etiolated 2; *prl1*, pleiotropic regulatory locus 1; P5CS, $\Delta^1$-pyrroline-5-carboxylate synthase; PDH, proline dehydrogenase

## Introduction

In response to osmotic stress stimulated by drought, salt, cold and abscisic acid (ABA), proline accumulates in many plant species. Proline performs an important function as protective compatible osmolyte in scavenging of free radicals and facilitating a correction of altered redox potential by replenishment of the $NADP^+$ supply (Hare *et al.*, 1999; Hasegawa *et al.*, 2000). Due to down-regulation of ornithine-$\delta$-aminotransferase, proline is directly synthesized from glutamate rather from ornithine under stress conditions. The glutamate-derived proline biosynthesis pathway includes a bifunctional $\Delta^1$-pyrroline-5-carboxylate synthase (P5CS) enzyme that phosphorylates and reduces glutamate to glutamyl-5-semialdehyde (G5SA), which is spontaneously converted to $\Delta^1$-pyrroline-carboxylate (P5C). The latter two intermediates are reduced to proline by $\Delta^1$-pyrroline-carboxylate reductase (P5CR, Delauney and Verma, 1993). The rate-limiting step in this pathway is represented by the $\gamma$-glutamyl kinase activity of P5CS, which is sensitive to feedback inhibition by relatively low levels of proline (Zhang *et al.*,

1995). During stress recovery, proline is converted to $\Delta^1$-pyrroline-5-carboxylate (P5C) by proline dehydrogenase (PDH) and subsequently to glutamate by P5C-dehydrogenase (P5CDH). Proline accumulation is thought to result from simultaneous activation of biosynthesis and inactivation of degradation pathways during stress.

P5CS is encoded by duplicated *P5CS1* and *P5CS2* genes, which show different temporal and spatial regulation in *Arabidopsis.* In most organs *P5CS1* is transcribed at much higher levels than *P5CS2*, which is preferentially expressed in dividing tissues and cell cultures stimulated by ABA and salt stress (Strizhov *et al.*, 1997; Yoshiba *et al.*, 1999). Salt induction of proline accumulation and *P5CS* gene expression is inhibited in the ABA-deficient *aba1* and ABA-insensitive *abi1 Arabidopsis* mutants (Savoure *et al.*, 1997; Strizhov *et al.*, 1997; Nambara *et al.*, 1998). However, expression of the *P5CS* genes is unaffected by the *abi2* and *abi3* mutations suggesting the existence of two parallel ABA signaling pathways (Shinozaki and Yamaguchi-Shinozaki, 2000). ABA induction of *P5CS1* represents a primary transcriptional response, whereas transcriptional activation of *P5CS2* by ABA is reduced by the protein synthesis inhibitor cycloheximide (Strizhov *et al.*, 1997). Regulation of *P5CS2* also involves an ABA-independent signaling pathway. Proline accumulation during cold stress thus correlates with the induction of *P5CS2* by ABA-independent expression of CBF3/DREB1a transcriptional activator (Gilmour *et al.*, 2000). Hypersensitivity to salt stress caused by the *sos1* (salt overly sensitive) mutation of a plasma membrane $Na^+/H^+$ antiporter enhances both proline accumulation and expression of *P5CS* genes (Liu and Zhu, 1997). *SOS1* is the first element of a recently established signaling cascade that includes SOS2, an *Arabidopsis* homologue of the yeast Snf1 sucrose non-fermenting protein kinase, and SOS3, a $Ca^{2+}$-binding protein showing similarity to the regulatory subunits of calcineurin-type protein phosphatases (for review see Zhu, 2001). How the SOS pathway is integrated into proposed pathways of osmotic (i.e. drought, ABA, salt and cold) stress signaling is however so far unclear (Shinozaki and Yamaguchi-Shinozaki, 2000).

In comparison to P5CS, less is known about the regulation of mitochondrial PDH and P5CDH enzymes that are encoded by single-copy nuclear genes in *Arabidopsis.* Both *PDH* and *P5CDH* genes are activated by proline during recovery from osmotic stress, albeit with different kinetics. However, *PDH* is inhibited by drought, salt, polyethylene glycol, glucose and sucrose treatments, whereas *P5CDH* is induced by salt, glucose and sucrose (Kiyoshue *et al.*, 1996; Verbruggen *et al.*, 1996; Deuschle *et al.*, 2001). *PDH* expression is de-repressed in the reduced sugar response mutant *rsr1*, which responds to proline feeding by P5C accumulation and subsequent induction of cell death (Hellmann *et al.*, 2000). Salt stress and feeding with glucose or sucrose can ameliorate the toxic effect of P5C accumulation. Thus far, it is unknown whether inhibition of *PDH* and induction of *P5CDH* genes by salt stress are mediated by ABA-dependent or -independent signaling pathways.

The data described here show that ABA and salt stress strongly activate *P5CS1*, weakly stimulate *P5CS2*, and down-regulate the expression of *PDH* gene in light-grown *Arabidopsis* plants. Dark adaptation inhibits proline accumulation and *P5CS1* transcription, but leads to inhibition of *P5CS2* and activation of *PDH* only in shoots. Treatment of light-grown plants with the steroid hormone brassinolide efficiently inhibits the induction of *P5CS1* transcription by ABA and salt stress, but fails to stimulate *PDH.* The *Arabidopsis prl1* and *det2* mutations, causing ABA hypersensitivity and brassinosteroid deficiency (Németh *et al.*, 1996; Li *et al.*, 1996) respectively, result in a simultaneous enhancement of proline accumulation and *P5CS1* transcription, but do not cause a coordinately enhanced down-regulation of *PDH* expression during ABA and salt induction. These data support the conclusion that proline accumulation in response to ABA and salt stress is principally controlled by the light-dependent induction of *P5CS1* gene expression, which is inhibited by brassinosteroid signaling in *Arabidopsis.*

## Materials and methods

### *Plant material and growth conditions*

Wild-type (ecotype Col-0), *prl1* (Németh *et al.*, 1998), and *det2* (Li *et al.*, 1996) mutant *Arabidopsis* seedlings were germinated and grown in MSAR seed medium as described (Koncz *et al.*, 1994) in controlled growth chambers at 24 °C under 150 $\mu$E $m^{-2}s^{-1}$ irradiance using a 8 h light/16 h dark cycle. Stress treatments described in Figures 1–5 were performed with 3-week old wild-type seedlings that were transferred into liquid MSAR seed medium containing either 200 mM NaCl or 10 $\mu$M ABA. When subjected to brassinosteroid treatment, the seedlings were pre-incubated

with 0.1 $\mu$M 24-*epi*-brassinolide for 3 days before addition of either NaCl or ABA. In each case, control plants were similarly treated in MSAR medium. Stress treatments shown in Figure 6 were carried out with 3-week old wild-type, *prl1* and *det2* mutant seedlings that were transferred from solid MSAR seed medium containing 15 mM sucrose for 24 h on solid MSAR medium supplemented with either 150 mM NaCl or 10 $\mu$M ABA. In these experiments, control plants were similarly transferred to fresh MSAR seed medium. For dark adaptation, plants grown under light-dark cycles were transferred to continuous darkness for 1–5 days and subjected to stress treatments in the dark. Plant materials were harvested 24 h after application of stress treatments and stored in liquid nitrogen.

*Vector constructions and plant transformation*

Characterization of *P5CS1* and *P5CS2* genes has been described earlier (Strizhov *et al.*, 1997). Promoter regions of *P5CS1* (GenBank gi|6598383, between positions 45500 and 48720) and *P5CS2* (GenBank gi|7263547, between positions 17110 and 19150) genes were isolated by PCR amplification and cloned in pBluescript $SK^+$ to determine their nucleotide sequence. To construct *P5CS-GUS* reporter genes, an *Eco*RI-*Xba*I fragment of 3.0 kb, carrying the *P5CS1* promoter, and a *Hin*cII-*Kpn*I fragment of 2.0 kb, carrying the *P5CS2* promoter, were cloned into corresponding cleavage sites of *Agrobacterium* binary vector pPR97 upstream of a promoterless *uidA*(*GUS*) reporter gene (Szabados *et al.*, 1995). These plasmid constructs were introduced into *Arabidopsis* by *Agrobacterium*-mediated *in planta* transformation as described (Bechtold *et al.*, 1993). The primary ($T_1$) transformants were selected on kanamycin (100 mg/l) containing MSAR seed medium and subjected to self-pollination. Plants carrying single copy T-DNA insertions in homozygous form in the $T_2$ generation were identified and propagated. GUS-expressing transgenic lines, carrying the *P5CS1-GUS* and *P5CS2-GUS* reporter constructs were crossed with a homozygous *prl1* mutant (Németh *et al.*, 1998). After selection and self-pollination of kanamycin-resistant $F_1$ lines, kanamycin-resistant $F_2$ progeny homozygous for the *prl1* mutation (which was marked by a T-DNA insertion carrying a selectable hygromycin resistance gene) was isolated and selfed to screen for lines carrying single-copy pPR97 T-DNA insertions with the *P5CS1-GUS* and *P5CS2-GUS* reporter genes in homozygous form.

*Northern RNA hybridizations*

Total RNA was extracted from 3-week old seedlings as described (Pawlowski *et al.*, 1994). The RNA samples were size separated in denaturing agarose gels and blotted onto nylon membranes (Amersham Hybond $N^+$). The hybridizations were performed with $^{32}$P-labeled gene-specific DNA probes prepared by PCR amplification of *PDH* cDNA (Hellmann *et al.*, 2000), and unique 3′-untranslated sequences of *P5CS1* and *P5CS2* cDNAs (Strizhov *et al.*, 1997). The gene-specific *P5CS1* and *P5CS2* hybridization probes were PCR-amplified with the primer pairs P5C1A (5′-AAACAAGACTTCCGAGTGTGTG-3′), P5C1B (5′-GGTAGCTTACAATGACAAGAAGAG-3′), P5C2A (5′-GTCTTACAAAGGACAGAGGCTG-3′), and P5C2B (5′-AACATTTCACTATTATACAAG ACCAC-3′). An ubiquitin cDNA was used as control hybridization probe. Quantitative analysis of hybridization signals was performed with a PhosphoImager 445SI (Molecular Dynamics), and evaluated with the Image QuaNT (version 4.1) and NIH Image 1.60 computer programs.

*GUS reporter enzyme assays and measurement of free proline content*

Free proline concentration in plant tissues was determined according to Bates (1973). Quantitative measurement of GUS enzyme activity was performed with a fluorimetric enzyme assay as described (Jefferson, 1987). Each experiment was performed in triplicates and repeated three times.

## Results

*Inhibition of proline accumulation and P5CS1 expression in dark-adapted plants*

In response to osmotic stress induced by drought, salt, cold or abscisic acid (ABA) treatments proline accumulates as protective osmolyte in many plant species (Hare *et al.*, 1999). Determination of free proline content in three weeks old *Arabidopsis* plants grown under short-day conditions showed that the concentration of free proline increased from an average of 15 mM to 35 and 48 mM in response to 24 h treatment with either 10 $\mu$M ABA or 200 mM NaCl, respectively (Figure 1).

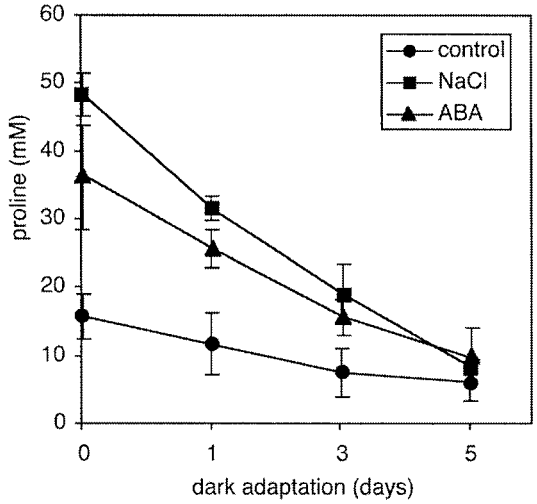


*Figure 1.* Light dependence of proline accumulation induced by salt and ABA treatments in *Arabidopsis* seedlings. Three-week old light-grown *Arabidopsis* plants were subjected to dark adaptation for 1 to 5 days before treatment with either NaCl (200 mM) or ABA (10 $\mu$M) for 24 h before determination of free proline content. Values represent means of three independent measurements. Bars indicate standard deviations.

The induction of proline accumulation by ABA and salt stress was however inhibited in plants subjected to dark treatment. After 5 days of dark adaptation the concentration of free proline decreased below 10 mM in untreated control plants and proline accumulation could be stimulated by neither ABA nor salt treatment suggesting that light signaling plays an important role in the regulation of either proline biosynthesis or proline degradation, or both.

The rate-limiting step of proline biosynthesis from glutamate is controlled by $\Delta^1$-pyrroline-5-carboxylate synthase (P5CS) encoded by functionally redundant *P5CS1* and *P5CS2* genes (Zhang *et al.*, 1995; Strizhov *et al.*, 1997), whereas the first step of proline degradation is regulated by a mitochondrial proline dehydrogenase encoded by a nuclear *PDH* gene in *Arabidopsis* (Kiyoshue *et al.*, 1996; Verbruggen *et al.*, 1996). Monitoring the steady-state levels of *P5CS* and *PDH* mRNAs by northern hybridization, with an ubiquitin (*UBI*) probe as internal control, revealed a striking 4- to 8-fold increase of *P5CS1* transcript levels in shoots and roots of light-grown plants treated with either 200 mM NaCl or 10 $\mu$M ABA for 24 h (Figure 2). In comparison, however, only minor alterations (i.e. a maximum of 1.5-fold increase in shoots and no induction in roots) were observed in the amounts of *P5CS2* mRNA under these conditions. By contrast, salt stress caused a 2- to 3-fold reduction, whereas ABA treatment led to 30–40% decrease in steady-state levels of *PDH* mRNA in shoots and roots. Proline accumulation induced by salt in light grown plants thus appeared to correlate with simultaneous up-regulation of *P5CS1* and down-regulation of *PDH* gene expression.

Dark adaptation of plants for 3 days resulted in 3- to 4-fold reduction of ABA- and salt-induced *P5CS1* expression in shoots and led to a 30–40% decrease of *P5CS1* mRNA levels in roots. Dark treatment also prevented weak induction of *P5CS2* expression in shoots, but slightly stimulated *P5CS2* in roots (Figure 2). In comparison, steady-state levels of *PDH* mRNA increased 1.5- to 2-fold in dark-adapted shoots of control, as well as ABA- and salt-induced plants. A similar increase in *PDH* transcript levels was observed in roots of ABA- and salt-treated, but not in control, dark-adapted plants. Inhibition of ABA- and salt-induced proline accumulation by dark treatment was thus accompanied by a clear inhibition of *P5CS1* transcript accumulation and simultaneous increase of *PDH* expression.

### *Induction of* P5CS1 *transcription by ABA and salt stress is inhibited by brassinosteroid*

To demonstrate that changes observed at the level of transcript accumulation reflected alterations in transcriptional regulation and not transcript stability of *P5CS* genes, similar induction experiments were performed with three weeks old transgenic plants carrying *uidA*(*GUS*) reporter genes under the control of *P5CS* promoters. Despite a relative stability of GUS reporter enzyme, the determination of $\beta$-glucuronidase specific activities in leaves of light grown plants indicated an about 3-fold higher activity of *P5CS1* promoter than in dark-adapted plants in the absence of ABA and salt induction (for comparison to 2-fold difference in *P5CS1* transcript levels, see Figure 2). However, dark treatment caused only a maximum of 50% reduction in the basal activity of *P5CS1-GUS* reporter in the roots. In both light and dark similar activities of *P5CS2-GUS* reporter were detected without ABA and salt induction in the shoots (Figure 3). By contrast, *P5CS2-GUS* activities in roots were about 2-fold higher in dark-adapted than in light-grown plants corresponding to a minor increase observed in *P5CS2* transcript levels (Figure 2).

*Arabidopsis* mutations causing brassinosteroid deficiency result in transcriptional de-repression of certain light-regulated genes in the dark and enhance the expression of some stress inducible genes in the light

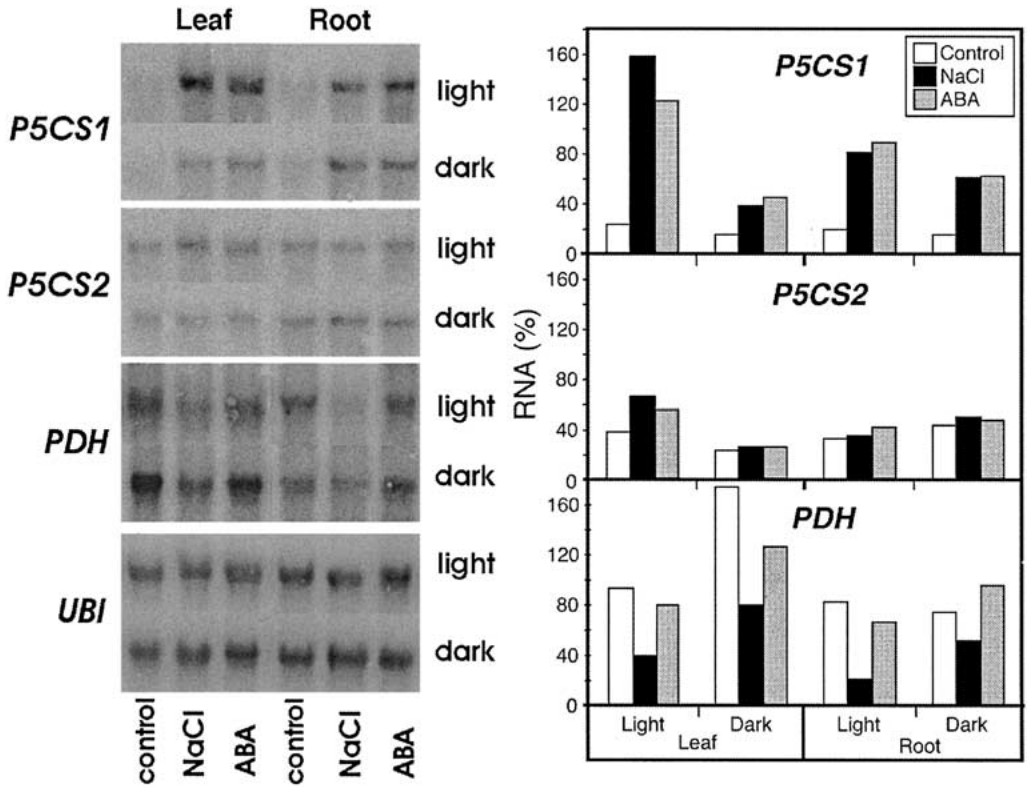


*Figure 2.* The effect of dark adaptation on the regulation of steady-state *P5CS1*, *P5CS2* and *PDH* transcript levels with and without induction by salt and ABA. Three-week old seedlings were transferred for 3 days into the dark, and then incubated in MSAR medium in the dark with either 200 mM NaCl or 10 $\mu$M ABA. Control samples were similarly treated in the light. Total RNA was extracted from leaves and roots separately, and then subjected to northern blotting and hybridization with $^{32}$P-labeled gene-specific *P5CS1*, *P5CS2*, *PDH* and control ubiquitin (*UBI*) probes. Quantitative evaluation of hybridization signals was performed by phosphorimager analysis with Image QuaNT (version 4.1) software. Bars in the schematic presentation to the right indicate the ratio of signals detected with gene specific probes (d1) and corresponding signals obtained with the ubiquitin control probe (d2) in values of RNA(%) = 100 × d1/d2.

(Szekeres *et al.*, 1996; Li *et al.*, 1996). To test whether brassinolide, the end product of plant steroid hormone biosynthesis pathways, would also control the activity of *P5CS* promoters, 3-week old light-grown plants carrying the *GUS* reporter constructs were treated for 3 days with 24-*epi*-brassinolide (BR, 0.1 $\mu$M) in either light or dark. Subsequently, brassinolide-treated and control untreated plants were subjected to induction by either 10 $\mu$M ABA or 200 mM NaCl for 24 h to compare the specific GUS enzyme activities in shoots and roots. In light-grown plants ABA and salt treatments resulted in a 2.5- to 3-fold induction of *P5CS1-GUS* activity in shoots and roots, which was not detectable in plants subjected either to dark adaptation, or brassinolide treatment in the light or dark. Whereas brassinolide mimicked the inhibitory effect of dark adaptation on the light-regulated *P5CS1-GUS* reporter gene, it did not alter the basal activity of *P5CS2-GUS* gene in the shoots, but prevented its induction by ABA and salt. As dark adaptation, brassinolide treatment of light- and dark-grown plants resulted in about 2-fold increase of *P5CS2-GUS* reporter activity in roots, which was not further elevated by either ABA or salt treatment (Figure 3).

### *Enhancement of ABA- and salt-induced proline accumulation and* P5CS1 *transcription by mutations causing brassinosteroid deficiency and ABA hypersensitivity*

To test whether alterations in ABA and brassinosteroid regulation cause also simultaneous changes in proline accumulation and *P5CS1* expression, first the levels of *P5CS1*, *P5CS2* and *PDH* mRNAs were compared in wild-type, *prl1* and *det2* mutant seedlings by northern hybridization (Figure 4). In comparison to wild type, a 1.6- to 1.8-fold increase of total *P5CS1* transcript levels was observed in response to ABA and salt

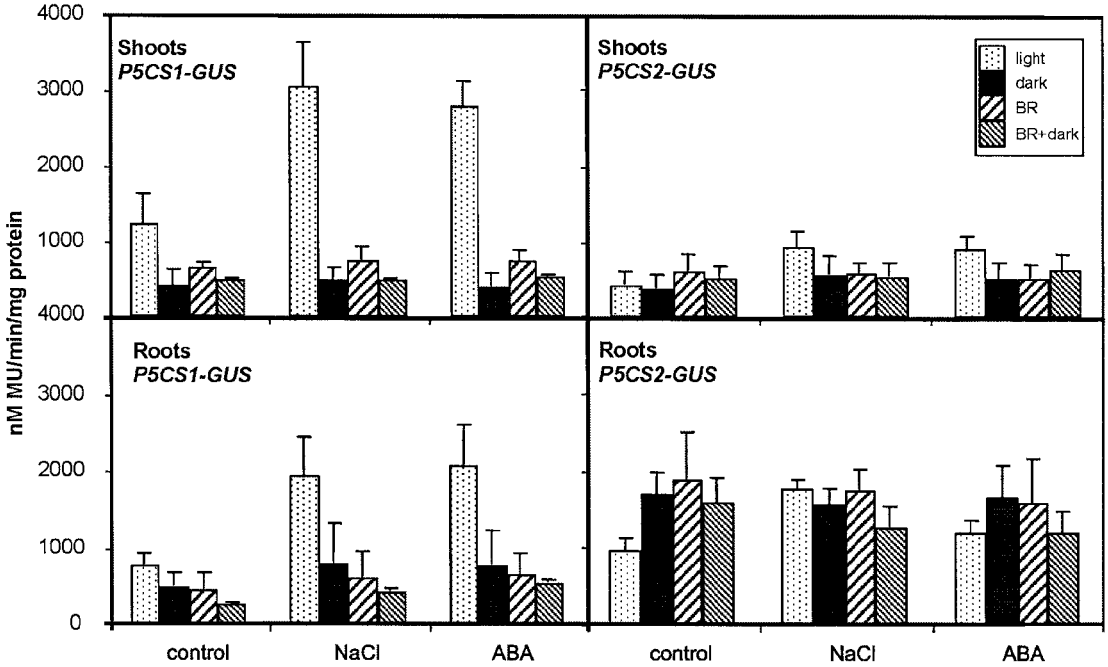


*Figure 3.* Regulation of activity of *P5CS1-GUS* and *P5CS2-GUS* reporter genes by dark adaptation and brassinolide treatment in shoots and roots of wild-type *Arabidopsis* seedlings with or without induction by NaCl and ABA. Three-week old light-grown seedlings were treated in MSAR medium with either 200 mM NaCl or 10 $\mu$M ABA for 24 h in the light or dark, with or without pre-treatment for 3 days with 0.1 $\mu$M brassinolide (BR). Columns show the means of specific GUS activities determined by fluorometric assays in three independent experiments. Bars indicate standard deviation.

induction in seedlings that carried a mutation in the *pleiotropic regulatory locus 1* (*prl1*) causing hypersensitivity to ABA (Németh *et al.*, 1998). A similar moderate increase of *P5CS1* mRNA levels was detected in the ABA-induced *det2* mutant, which is deficient in brassinosteroid biosynthesis (Li *et al.*, 1996). As in the wild type, pretreatment of seedlings with 0.1 $\mu$M 24-*epi*-brassinolide for 3 days before ABA and salt induction drastically reduced the *P5CS1* transcript levels in the *det2* and *prl1* mutants indicating that these mutations did not alleviate the inhibitory effect of brassinosteroid. Transcript levels of *P5CS2*, which showed some increase in the *det2* mutant, were also reduced about 2-fold in brassinolide-treated plants. Although *PDH* showed different regulation in shoots and roots (data not shown), the hybridization analysis indicated that brassinolide did not increase, but rather decreased the total amount of *PDH* mRNA in wild-type plants and, albeit to less extent, in the *prl1* and *det2* mutants. Subtle changes in the total amount of *PDH* mRNA in addition reflected a reduction of *PDH* transcript levels in the uninduced *prl1* and ABA-treated *det2* mutants.

To confirm that the RNA hybridization data indeed reflected a significant alteration in *P5CS1* transcription, the regulation of *P5CS1-GUS* reporter gene was assayed in the *prl1* mutant. As compared to wild type, 1.5- to 2-fold higher GUS enzyme activities were detected in shoots and roots of the *prl1* mutant after 24 h of treatment with either 10 $\mu$M ABA or 150 mM NaCl (Figure 5). A similar difference in the activity of *P5CS1-GUS* reporter between wild type and *prl1* mutant was observed when the plants were treated with brassinosteroid before ABA and salt induction as described above.

To determine whether enhancement of *P5CS1* expression would result in a higher level of proline accumulation in the *prl1* and *det2* mutants, the concentration of free proline was compared in 3-week old wild-type, *prl1* and *det2* plants, which were transferred from solid MSAR medium containing 15 mM sucrose (Koncz *et al.*, 1994) for 24 h on MSAR medium supplemented with either 150 mM NaCl or 10 $\mu$M ABA (Figure 6). The exposure of roots to ABA and salt treatment under these conditions resulted in 2- and 5-fold increase of proline concentration, respectively, which was on an average 18 mM in control wild-type plants. The basic level of free proline reached 30 mM in the uninduced *det2* mutant

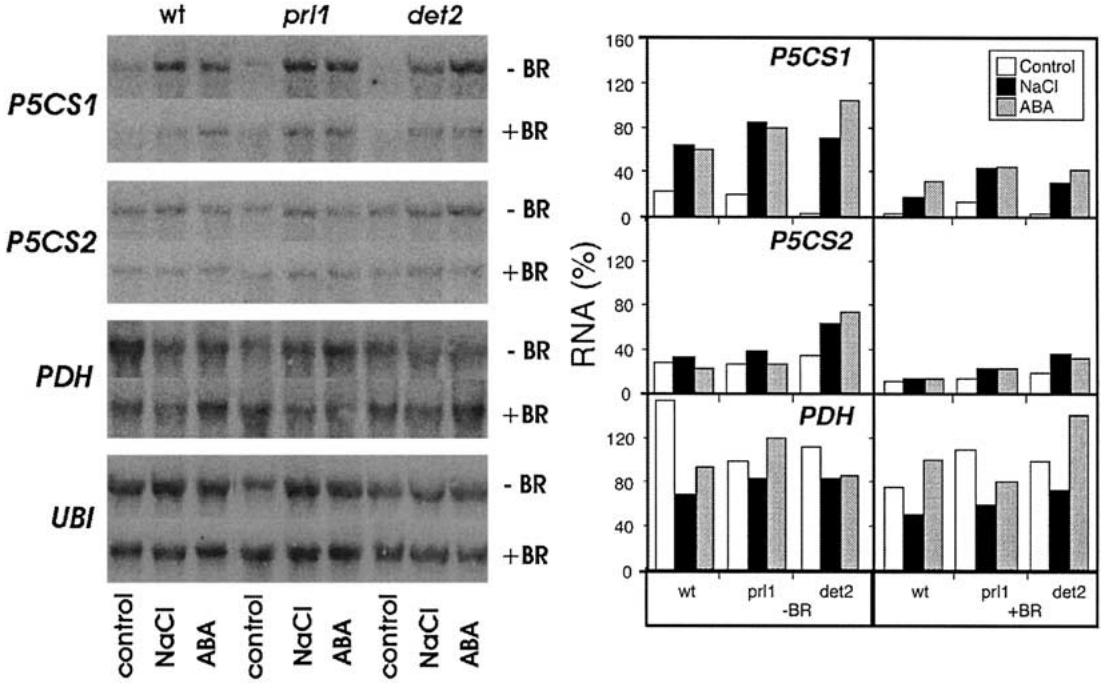


*Figure 4.* Analysis of steady-state *P5CS1*, *P5CS2* and *PDH* transcript levels in brassinolide treated wild-type, *prl1* and *det2* mutant seedlings. Three-week old light-grown plants were pre-incubated with or without 0.1 $\mu$M brassinolide (BR) for 3 days in MSAR liquid medium in the light, then treated with either 200 mM NaCl or 10 $\mu$M ABA for 24 h. Control samples were similarly cultured in MSAR medium. Samples of total RNA isolated from whole seedlings were hybridized with gene-specific *P5CS1*, *P5CS2*, *PDH* and control ubiquitin (*UBI*) probes. The hybridization data were evaluated by phosphorimager analysis and presented in the column diagram to the right as described in Figure 2.

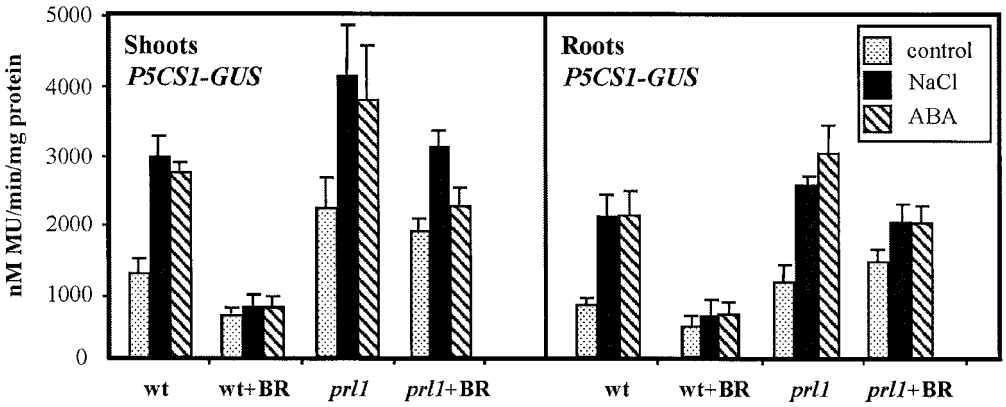


*Figure 5.* The *P5CS1-GUS* reporter gene shows de-repressed expression in the *prl1* mutant. Three-week old light-grown wild-type and *prl1* mutant seedlings were treated in MSAR medium with either 200 mM NaCl or 10 $\mu$M ABA for 24 h in the light or dark, with or without pre-treatment for 3 days with 0.1 $\mu$M brassinolide (BR). Bars show the means of specific GUS activities determined by fluorometric assays in three independent experiments. Standard deviation is indicated above the bars.

that responded similarly by 2- and 5-fold increase of proline accumulation to ABA and salt treatments, respectively. In comparison, the proline concentration was close to 50 mM in the *prl1* mutant, which was further increased to over 300 mM by salt and to about 90 mM by ABA treatment. As seen in dark-adapted plants, alterations in the level of *AtP5CS1* expression thus correlated with similar changes in proline accumulation also in the ABA hypersensitive *prl1* and brassinosteroid-deficient *det2* mutants.

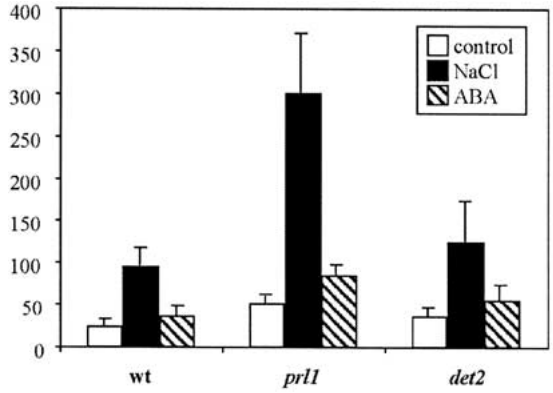


*Figure 6.* Enhanced proline accumulation in the *prl1* and *det2 Arabidopsis* mutants. Three-week old wild-type, *prl1* and *det2* mutant seedlings were transferred from solid MSAR seed medium for 24 h on MSAR medium supplemented with either 150 mM NaCl or 10 $\mu$M ABA before determination of free proline content in three independent experiments. Standard deviations are indicated above the bars.

## Discussion

Proline accumulation during osmotic stress in plants is controlled by coordinate induction of biosynthesis and inhibition of degradation pathways. Previous studies demonstrated that duplicated *P5CS1* and *P5CS2* genes code for $\Delta^1$-pyrroline-5-carboxylate synthase enzymes, which catalyze the rate-limiting step of proline biosynthesis in *Arabidopsis.* These studies also revealed that salt induction of *P5CS1* transcription is mediated by an ABA-dependent early transcriptional response, which is inhibited in the ABA-deficient *aba1* and ABA-insensitive *abi1* mutants (Yoshiba *et al.*, 1995, 1999; Strizhov *et al.*, 1997), and showed that *P5CS2* is regulated by both ABA-dependent and -independent signaling pathways (Savoure *et al.*, 1997; Gilmour *et al.*, 2000). Recent characterization of *Arabidopsis* proline dehydrogenase and $\Delta^1$-pyrroline-5-carboxylate dehydrogenase genes, on the other hand, uncovered that *PDH* is inhibited, whereas *P5CDH* is stimulated by salt indicating that only PDH could play a role in the inhibition of proline degradation during salt stress (Hellmann *et al.*, 2000; Deuschle *et al.*, 2001).

To determine whether induction of proline accumulation by salt stress and ABA indeed correlates with a coordinate inhibition of *PDH* and induction of *P5CS* gene expression, we have searched for signaling cues that inhibit this process. We have found that proline accumulation is inhibited in dark-adapted plants suggesting that either light signaling, or indirectly a light-dependent metabolic or hormonal signaling pathway, plays a role in the control of proline metabolism. Analysis of steady-state transcript levels in light-grown and dark-adapted plants indicated that light is essential for both basal and induced transcription of *P5CS1*, which appears to play a major role in controlling proline accumulation. In comparison, dark-adaptation appeared to cause less dramatic changes in tissue-specific regulation of *P5CS2*. In contrast to inhibition of *P5CS* genes, dark adaptation significantly enhanced *PDH* expression in shoots, but not in roots. Whereas ABA and salt stress resulted in similar induction of the *P5CS1* gene, the expression of *PDH* gene was efficiently inhibited by salt, but to a lesser extent by ABA. These data suggested that the induction of proline accumulation by ABA and salt stress is modulated by up-regulation of *P5CS1* and down-regulation of *PDH* genes in *Arabidopsis.*

A principal role for *P5CS1* in the regulation of proline accumulation was suggested by the fact that the steroid hormone brassinolide efficiently inhibited *P5CS1* transcription, but did not result in coordinate up-regulation of *PDH* expression. The fact that induced levels of both *P5CS1* transcript and *P5CS1-GUS* reporter activity were simultaneously reduced in roots and shoots of dark-adapted and brassinosteroid-treated plants suggested that the observed light dependence of *P5CS1* induction could implicate a metabolic or hormonal factor. Although ABA is required for salt induction of *P5CS1* expression (Strizhov *et al.*, 1997), it is unlikely that ABA would correspond to this light-dependent regulatory factor, as ABA concentrations increase, rather than decrease in dark-adapted *Arabidopsis* plants (Weatherwax *et al.*, 1996). In addition, the induction of *P5CS1* by ABA is also inhibited by dark adaptation and brassinosteroids. By contrast, sucrose and glucose are likely candidates for being light-dependent metabolic regulators, as *P5CS1* is induced, whereas *PDH* is inhibited by externally provided sucrose. These genes also show different regulation in the reduced sucrose response mutant *rsr1* (Hellmann *et al.*, 2000).

An intimate cross-talk between ABA and sugar signaling was recently uncovered by the finding that the *Arabidopsis* glucose and sucrose-insensitive mutations *gin1/sis4* and *gin6/sis5* are allelic with the *aba2* and *abi4* mutations that cause defects in ABA biosynthesis and signaling, respectively (for review see Gibson, 2000). Whereas negative regulation of *PDH* by ABA signaling requires further studies, it is known that the *aba1* and *abi1* mutations abol-

ish salt induction of *P5CS1*. Thus, the finding that *P5CS1* expression is inducible by sucrose and glucose (Hellmann *et al.*, 2000) apparently contradicts the observation that neither the *aba1* nor the *abi1* mutation confers altered glucose sensitivity (Arenas-Huertero *et al.*, 2000). However, these results lend further support to a model, which suggests the existence of two parallel ABA-dependent signaling pathways (Shinozaki and Yamaguchi-Shinozaki, 2000). According to this model, salt stress is expected to stimulate an ABA-signaling pathway, which requires the *ABA1* and *ABI1* functions and triggers transcriptional activation of *P5CS1* by an ABA-responsive promoter element (ABRE). The fact, that both ABA and salt induction of *P5CS1* transcription is inhibited by brassinolide in light-grown plants suggests that steroid hormones may negatively regulate this common salt and ABA response pathway. Alternatively, brassinolide may inhibit the light or sugar (or both) regulated maintenance of basal *P5CS1* transcription, which is essential for further induction by salt and ABA.

Brassinosteroid treatment decreases the basal activity of *P5CS1* and *P5CS1-GUS* genes in shoots and roots of wild-type plants (see Figures 4 and 5). We have shown that the *prl1* mutation, which causes hypersensitivity to ABA, sucrose and glucose (Németh *et al.*, 1998), results in a 2-fold increase of basal activity of *P5CS1-GUS* reporter (Figure 5). This increased basal activity of *P5CS1-GUS* gene cannot be fully suppressed by brassinosteroid in the *prl1* mutant as in the wild type. Nonetheless, following induction by ABA and salt, the *P5CS1-GUS* gene is down-regulated in the *prl1* mutant by brassinosteroid, but to a significantly lesser extent than in wild-type plants. These results show that brassinolide also inhibits the induction of *P5CS1* transcription by both ABA and salt stress in addition to reducing basal transcription of *P5CS1*. Further exploitation of *P5CS1* as reporter gene offers therefore a useful means for genetic dissection of control points where brassinosteroid regulation intercepts the light-dependent salt and ABA signaling pathways modulating proline accumulation in *Arabidopsis.*

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