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Hypersensitivity of an Arabidopsis Sugar Signaling Mutant toward Exogenous Proline Application[1]

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In transgenic Arabidopsis a patatin class I promoter from potato is regulated by sugars and proline (Pro), thus integrating signals derived from carbon and nitrogen metabolism. In both cases a signaling cascade involving protein phosphatases is involved in induction. Other endogenous genes are also regulated by both Pro and carbohydrates. Chalcone synthase (CHS) gene expression is induced by both, whereas the Pro biosynthetic Δ^1-pyrroline-5-carboxylate synthetase (P5CS) is induced by high Suc concentrations but repressed by Pro, and Pro dehydrogenase (ProDH) is inversely regulated. The mutant *rsr1-1*, impaired in sugar dependent induction of the patatin promoter, is hypersensitive to low levels of external Pro and develops autofluorescence and necroses. Toxicity of Pro can be ameliorated by salt stress and exogenously supplied metabolizable carbohydrates. The *rsr1-1* mutant shows a reduced response regarding sugar induction of *CHS* and *P5CS* expression. *ProDH* expression is de-repressed in the mutant but still down-regulated by sugar. Pro toxicity seems to be mediated by the degradation intermediate Δ^1-pyrroline-5-carboxylate. Induction of the patatin promoter by carbohydrates and Pro, together with the Pro hypersensitivity of the mutant *rsr1-1*, demonstrate a new link between carbon/nitrogen and stress responses.

Amino acids are key factors in metabolism and development of higher plants. Moreover, amino acids act as signaling molecules, controlling their own metabolism and the expression of a variety of genes (Lam et al., 1994; Kiyosue et al., 1996; Nielsen et al., 1998).

Under environmental stress conditions such as salt, cold, and drought stress, many plants accumulate compatible solutes such as Pro and Gly betaine. The function of Pro under stress conditions is not fully understood. The imino acid is discussed as a compatible osmolyte, which, in addition, might serve as a protectant of macromolecules or even as a scavenger of hydroxyl radicals (Wyn Jones et al., 1977; Schobert and Tschesche, 1978; Smirnoff and Cumbes, 1989). Furthermore, Pro can serve as a rapidly available source of nitrogen, carbon, and reduction equivalents during recovery from stress (Blum and Ebercon, 1976; Ahmad and Hellebust, 1988).

Principally, accumulation of Pro can be achieved in three different ways, (a) de novo synthesis in the affected cells (Rhodes et al., 1986; Voetberg and Sharp, 1991), (b) decreased degradation, or (c) specific transport systems that distribute Pro to the locations of need (Rentsch et al., 1996; Schwacke et al., 1999). Pro is synthesized in the cytosol, mainly from Glu via Δ^1-pyrroline-5-carboxylate (P5C), catalyzed by P5C-synthetase (P5CS) and P5C-reductase (P5CR) (Verbruggen et al., 1993; Savoure et al., 1995; Nanjo et al., 1999). Degradation of Pro to P5C takes place in mitochondria and is mediated by Pro dehydrogenase (ProDH) (Kiyosue et al., 1996). De novo synthesis, catabolism, and transport of Pro are highly regulated by both abiotic stress and cellular Pro concentrations. By means of feedback regulation, Pro represses expression of P5CS and induces ProDH expression (Verbruggen et al., 1993; Kiyosue et al., 1996; Peng et al., 1996). On the other hand, salt stress acts as an antagonist, overruling Pro-dependent regulatory mechanisms, and is capable of inducing expression of a Pro transporter (Kiyosue et al., 1996; Rentsch et al., 1996).

The accumulation of Pro under osmotic stress is often accompanied by an increase of the soluble sugar content (Larher et al., 1993; Pesci, 1993; Balibrea et al., 1997; Chen et al., 1998; Clifford et al., 1998). On the other hand, transgenic tobacco plants that accumulated high amounts of soluble carbohydrates due to ectopic expression of a yeast invertase had increased Pro content as well (Heineke et al., 1992). However, it is unclear whether the increase in Pro content was due to specific sugar responses or a response to osmotic stress. Larher et al. (1993) showed a specific increase of Pro by external supply of metabolizable carbohydrates, but not by the sugar alcohol mannitol. The identification of a mechanism that allows the plant to discriminate osmotic stress caused by utilizable sugars or other compounds would help to explain these observations. Such a mechanism will probably consist of sensors for metabolite concentrations or fluxes and subsequent signaling cascades that allow differential gene expression in response to different stresses (Roitsch, 1999).

To identify sensing and signaling pathways involved in metabolic control, Arabidopsis was transformed with the patatin class I promoter from potato (*Solanum tuberosum*) fused to a GUS reporter gene (Martin et al., 1997). In potato, patatin serves as the main storage protein in the tuber and the class I promoter is regulated by both carbohydrates and amino acids (Rocha-Sosa et al., 1989). In the transgenic

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Arabidopsis plants (which will be referred to as Pat(B33)-*Gus*), the patatin class I promoter is mainly active in the root and is also up-regulated by carbohydrates and Gln (Martin et al., 1997). This indicates a conserved regulatory pathway for sink-specific, metabolite-dependent gene expression present in Arabidopsis and in potato. After chemical mutagenesis, several *reduced sugar response* (*rsr*) mutants were identified (Martin et al., 1997).

We describe the regulation of the patatin class I promoter by Pro. In the mutant *rsr1-1*, neither sugar nor Pro are able to induce the patatin class I promoter. During analysis of Pro induction of the patatin promoter, it was found that even moderate concentrations of Pro are toxic for Arabidopsis in axenic culture. The mutant *rsr1-1* turned out to be hypersensitive to Pro and served as a convenient tool to study Pro toxicity. A more detailed investigation gave strong evidence that not Pro, but its degradation intermediate P5C causes toxicity. Regulation of the patatin promoter by sugar and Pro together with the Pro hypersensitivity of *rsr1-1* demonstrate a new link between carbon/nitrogen metabolism and stress response.

MATERIALS AND METHODS

Plant Growth: Tissue Culture

Arabidopsis L. Heynh., ecotype C24, Arabidopsis Pat(B33)-*Gus*, and the mutant *rsr1-1* (Martin et al., 1997) were grown in tissue culture on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962; purchased from DIFCO Laboratories, Detroit) supplemented with different carbohydrates and amino acids. Seeds were kept at 4°C for 48 h before sowing. Backcrossed seedlings were used for physiological analyses (Martin et al., 1997). Selection of hypersensitivity to Pro in the medium was performed by germinating seeds of ethyl methanesulfonate (EMS)-mutagenized Pat(B33)-*Gus* plants (Martin et al., 1997) on MS medium supplemented with 40 mM Pro and 30 mM Glc. Hypersensitive plants were rescued by transfer to plates with MS medium supplemented with 60 mM Suc (2MS).

Plant Analyses

Analysis of GUS Activity

Seedlings were grown on solidified MS medium supplemented with sugars or amino acids. Fluorimetric GUS assays were performed as described in Martin et al. (1997) in the presence of proteinase inhibitors (Boehringer Mannheim/Roche, Basel).

Extraction and Determination of Pro

Roots and fully expanded leaves derived from plants that had been cultured on MS medium supplemented with Glc and Pro were harvested around noon. Samples of 0.1 to 0.2 g fresh weight were ground in liquid nitrogen, soluble sugars were extracted twice with 200 μL of 80% (v/v) ethanol/10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0, once with 200 μL of 20% (v/v) ethanol/10 mM HEPES, pH 7.0, and once with 10 mM HEPES, pH 7.0, at 80°C for 30 min. Pro content was measured photometrically at 515 nm after incubation of 200 μL of extract with an equal volume of toluol/ninhydrin (Sigma, St. Louis) at 80°C for 1 h according to a modified method of Bates et al. (1973).

Inhibitor Assay and P5C Treatments

Plants were cultured on MS medium containing 30 mM Glc. Twenty-day-old plants were transferred to chambers (Weck round-rim jar 100), in which roots had contact with 10 mL of liquid MS medium. Pro was added after 2 h of pretreatment with water-soluble okadaic acid (0.4 μM in MS medium; Calbiochem-Novabiochem, San Diego). After 30 h, dissected roots were used for quantification of GUS activity. P5C was supplied as its 2,4-dinitrophenylhydrazine hydrochloride double salt (Sigma). As a control, 2,4-dinitrophenylhydrazine (Merck, Rahway, NJ) was used alone and no negative effects were observed. Incubations lasted up to 72 h.

RNA Gel-Blot Analyses

Thirty-day-old non-bolting plants cultured on solidified 2MS medium (60 mM Suc) were transferred to liquid medium as described for the inhibitor assays. Root and leaf material was collected separately. RNA extraction, gel electrophoresis, and blotting were done according to the method of Lehrach et al. (1977) and Logemann et al. (1987). cDNA clones were labeled by random priming. Hybridizations were carried out according to the method of Martin et al. (1997).

RESULTS

Pro Is a Potent Inducer of Pat(B33)-*Gus* Promoter Activity

The patatin class I promoter is inducible by Suc, Glc, and Glc analogs (Martin et al., 1997). Gln (12 mM) was also capable of triggering GUS expression in Pat(B33)-*Gus* plants, when Suc content in the medium was reduced to 3 mM (Martin et al., 1997). To investigate whether Gln induction is specific, the effects of citrulline, Pro, and Gln were compared. In the presence of 20 mM Glc, Gln weakly induced the patatin promoter in roots (Fig. 1). The highest GUS activity was detectable in plants cultured on medium containing Pro, whereas citrulline showed an intermediate effect. GUS activity was detectable not only in continuous culture on Pro-containing medium, but also after transfer of 20-d-old plants to liquid medium supplemented with Pro. However, short-term induction resulted in lower GUS activity in the roots (Fig. 2). In contrast, none of the treatments induced promoter activity in leaves.

To determine whether sugar and Pro induction act via overlapping or independent pathways, the sugar-signaling mutant *rsr1-1* was incubated in the presence of Pro. Short-term induction on liquid MS medium containing 200 mM Pro did not lead to an increase in GUS activity, demonstrating that *rsr1-1* is affected in both Pro

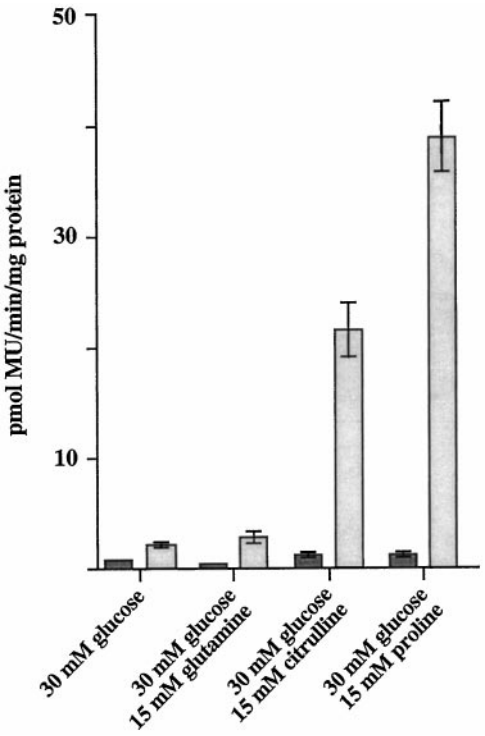

Figure 1. Regulation of the class I patatin promoter by amino acids. Pat(B33)-*Gus* plants were cultured on MS medium containing 30 mM Glc and 15 mM Gln, citrulline, or Pro. After 30 d, samples were harvested to quantify GUS activity in roots and leaves. In this and all subsequent diagrams each column represents the mean of five independent measurements. Error bars indicate SD. Dark-shaded bars, Leaf; light-shaded bars, root.

and carbohydrate-dependent regulation of the patatin class I promoter (Fig. 2).

Effect of Phosphatase Inhibitors on Pro Induction

Protein phosphatases are involved in the sugar-mediated regulation of storage protein expression, e.g. β-amylase, sporamin, and patatin (Takeda et al., 1994; H. Hellmann, unpublished data). To investigate the participation of protein phosphatases in the Pro-dependent regulation of the class I promoter, the phosphatase 2 and 2A inhibitor okadaic acid was used. Nanomolar concentrations of okadaic acid were potent in blocking the Pro-dependent response in Pat(B33)-*Gus* plants, indicating that protein phosphatases are involved in the induction by both Glc and Pro (Fig. 2).

Negative Effects of Exogenously Supplied Pro on Arabidopsis Growth

Pro serves as a compatible solute that under stress conditions accumulates in the cytosol to high amounts without harming the cell or negatively affecting cell metabolism (80–90 mM in potato leaves; Büssis and Heineke, 1998). Interestingly, moderate external Pro concentrations (15 mM Pro/30 mM Glc) were highly toxic to *rsr1-1* (Fig. 3, A and B). Under these conditions, the mutant was able to germinate and expand cotyledons, but already showed lesions 5 to 10 d post germination, and more than 90% of the plants did not develop primary leaves. Furthermore, root growth was strongly inhibited (Fig. 3, A and B). After 15 to 20 d, nearly all seedlings turned dark brown and died. When Pro was supplied as the sole nitrogen source, the effects were even more severe (data not shown). External supply of Pro in the absence of abiotic stress was also toxic for Pat(B33)-*Gus* and Arabidopsis wild type, but higher concentrations were needed (Fig. 3, D and E). Plants that were cultured on 40 mM Pro in the presence of 30 mM Glc developed necrotic and brown spots in the root, stem, and leaf tissues. The spots appeared first around vascular tissue and cell walls of the hypocotyl (Fig. 3D), but at later stages, most of the organs were affected (Fig. 3E). Comparable effects were observed in excised Arabidopsis leaves incubated for 48 h on agar plates supplemented with 200 mM Pro (Fig. 3F).

Necrosis and browning resembled the hypersensitive response to pathogens that is associated with accumulation of phenolic compounds and increased lignification. Plants cultured on Pro-containing medium emitted fluorescence in roots and leaves after excitation with UV (470 nm), indicating the presence of phenolic compounds. In leaves, fluorescence was first detectable as small spots that enlarged in heavily affected older leaves (Fig. 3, G–K). Root

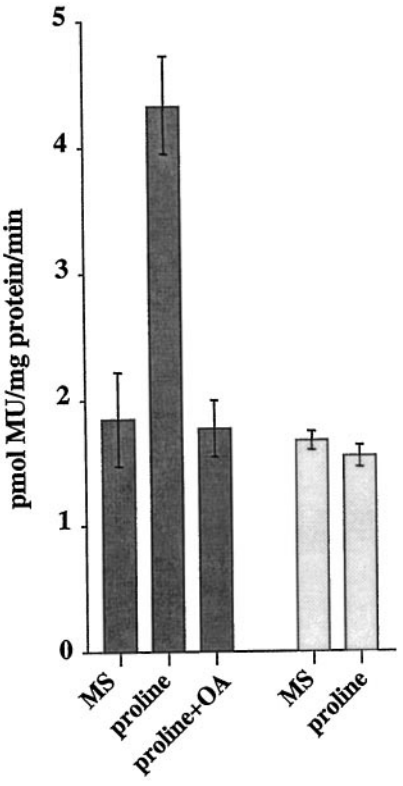

Figure 2. Quantification of GUS-activity in roots of Pat(B33)-*Gus* (dark-shaded bars) and *rsr1-1* (light-shaded bars) plants, cultured on MS medium containing 30 mM Glc. Samples were taken after 30 h of incubation time on 200 mM Pro in the presence or absence of 0.4 μM okadaic acid. Plants were pretreated for 2 h with the inhibitor before Pro was added.

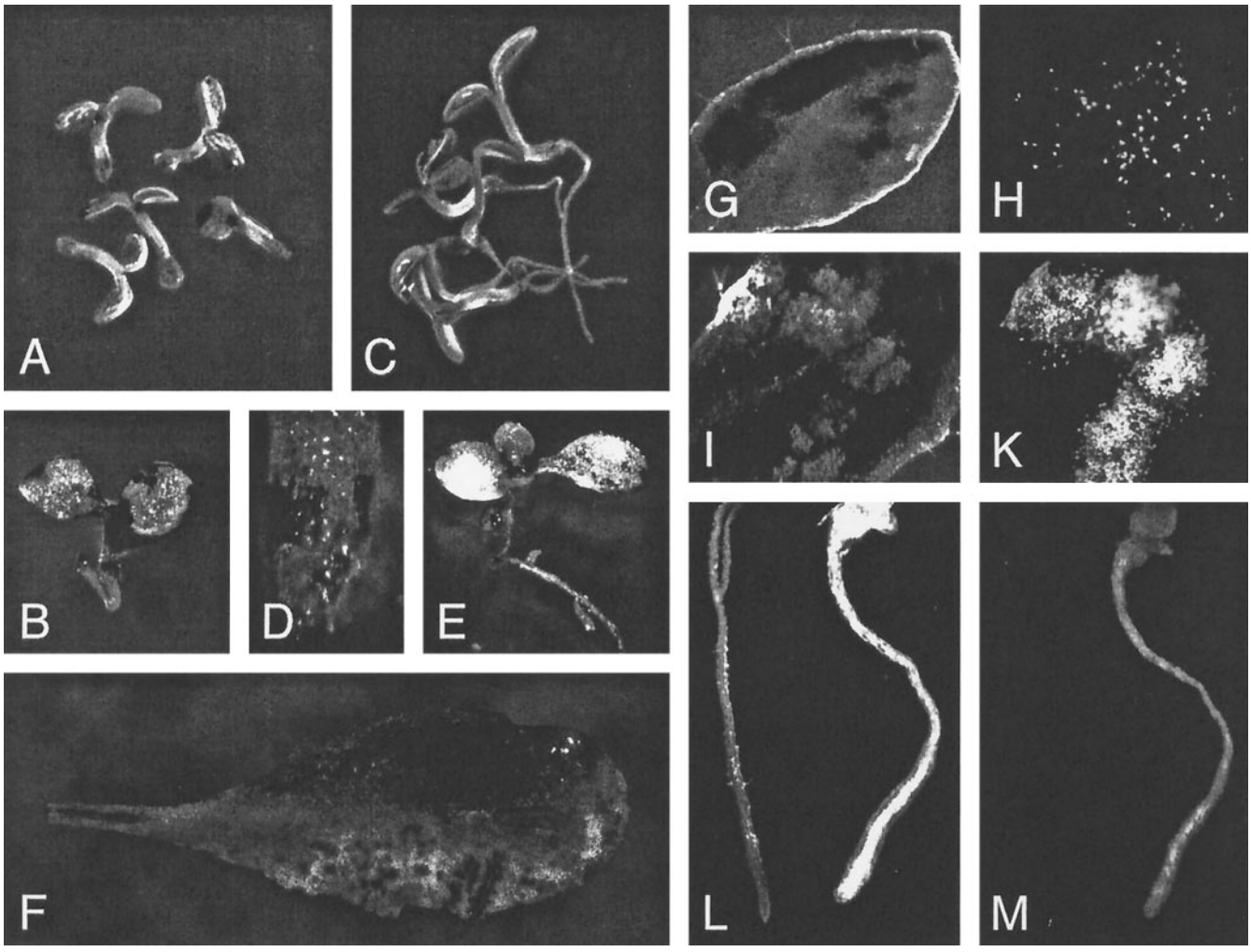

Figure 3. Toxicity of exogenously supplied Pro. The mutant *rsr1-1* is hypersensitive to Pro: After 9 d on MS medium containing 15 mM Pro and 30 mM Glc, extensive lesions were visible and root growth was almost completely inhibited (A). After 12 d the seedlings died (B). Under the same conditions, Pat(B33)-*Gus* showed almost no lesions after 9 d (C). On higher Pro concentrations (40 mM/30 mM Pro/Glc), Pat(B33)-*Gus* also showed lesions in various organs (D after 9 d; E after 15 d). Excised leaves of the Arabidopsis C24 wild type showed similar lesions when incubated for 48 h on agar plates containing 200 mM Pro (F). Prior to the appearance of necrotic tissue (G, I, and L), autofluorescent compounds accumulated (H, K, and M; excitation 470 nm). L and M, Left, Root of Pro-untreated plant; right, root of Pro-treated plant.

material showed intense fluorescence all over (Fig. 3, L and M). No comparable fluorescence was detected in leaves from plants cultured on MS medium in the absence of Pro (data not shown).

Reduction of Pro Toxicity by Salt Stress and Increasing External Glc Concentrations

The observed toxicity of Pro seems to contradict the beneficial effects reported for Pro under salt stress (Hare et al., 1999). Salt stress is accompanied by accumulation of Pro in many plant species due to the reduction of Pro catabolism and increased biosynthesis. In Arabidopsis, the key enzyme for Pro biosynthesis, P5CS, is up-regulated by salt stress, whereas the Pro-degrading enzyme ProDH is repressed (Hare et al., 1999). Since salt stress represses Pro degradation, it is possible that catabolism of Pro causes toxicity. To determine whether Pro toxicity can be reduced by the addition of salt, *rsr1-1* and Pat(B33)-*Gus* plants were cultured on media containing different NaCl concentrations. The presence of salt stress overcame Pro toxicity, since *rsr1-1* did not show any necrosis when cultured on 50 mM NaCl/15 mM Pro and 100 mM NaCl/15 mM Pro in the presence of 30 mM Glc (Fig. 4, D and F). In addition, the presence of Pro improved plant growth under high-salt conditions. In the presence of salt stress, Pro-dependent induction of the patatin promoter was inhibited. This indicated that Pro catabolism is also required for regulation of the patatin promoter (Fig. 5).

Since Pro and sugar signal transduction probably use overlapping pathways in the case of the patatin class I promoter, we tested whether Glc can also influence Pro toxicity. Plants were cultured on 30 and 120 mM Glc in the presence of 15 mM Pro. Increasing sugar concentrations led

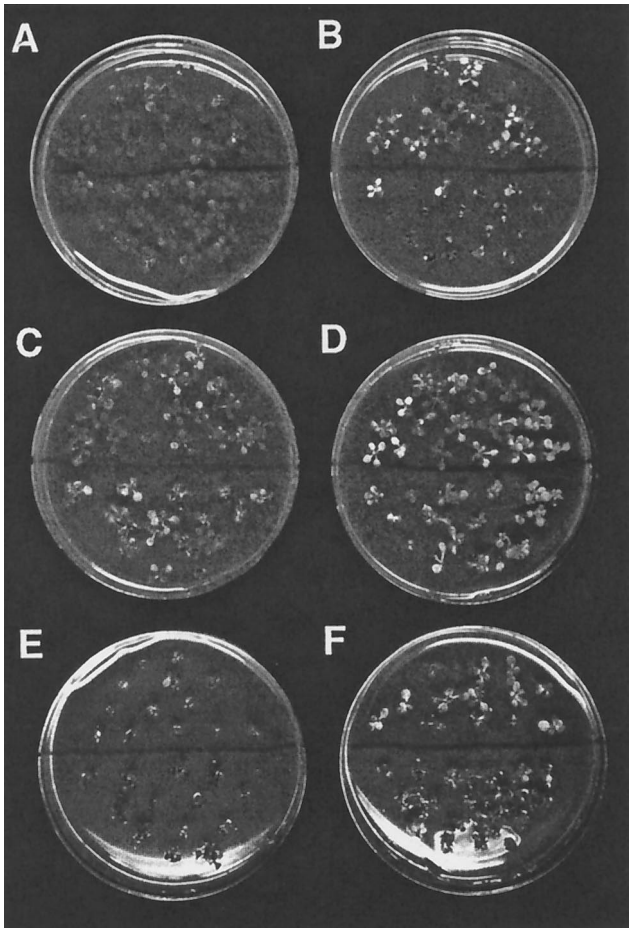

Figure 4. Amelioration of Pro toxicity by salt stress. Growth of Pat(B33)-*Gus* (upper halves of the plates) and *rsr1-1* (lower halves) on MS medium containing 20 mM Glc supplemented with different salt concentrations (A and B, no salt; C and D, 50 mM NaCl; E and F, 100 mM NaCl) in the presence (B, D, and F) or absence of Pro (A, C, and E).

to reduced toxicity of Pro (Fig. 6C). 3-*O*-Methyl-Glc (3Omeg), which is also able to induce the patatin promoter (Martin et al., 1997), could not equally substitute for Glc, since a combination of 30 mM Glc and 90 mM 3Omeg did not rescue *rsr1-1* (Fig. 6D). The amelioration of Pro toxicity by Glc but not by 3Omeg indicates the necessity to metabolize the imported Glc.

Increasing external Glc might lead to changes in internal Pro concentrations and thereby modify the strength of Pro toxicity. Determination of Pro content of plants cultured on Pro-free medium showed that endogenous Pro content was similar in Pat(B33)-*Gus* and *rsr1-1* (Fig. 6E). Both showed an increase in Pro content parallel to the rising external Glc concentrations (30–120 mM), but under the tested conditions total Pro content in *rsr1-1* stayed 50% to 60% below that measured in Pat(B33)-*Gus* (Fig. 6E). These findings show that hypersensitivity of *rsr1-1* toward Pro is not due to higher internal Pro concentrations and makes it unlikely that Pro itself causes the toxicity. Since the lower Pro content of *rsr1-1* can be caused by faster degradation, a toxic intermediate might be responsible for the hypersensitivity.

Damages Are Not Caused by Pro But by the Degradation Intermediate P5C

Pro is oxidized in a two-step process, first to P5C and further to Glu. Neither Glu nor Gln had inhibitory effects on growth of Pat(B33)-*Gus* and *rsr1-1* (data not shown). γ-Aminobutyric acid (GABA) degradation is similar to Pro degradation in that it also leads to production of Glu and reduction equivalents. However, even when 150 mM GABA was supplied as the sole source of nitrogen, no lesions were observed (data not shown). This indicates that Pro toxicity is not due to the overproduction of Glu or reduction equivalents.

In contrast, concentrations as low as 1 mM P5C were lethal for both Pat(B33)-*Gus* and *rsr1-1* within 3 d. The first signs of toxicity were observed as a reduction of chlorophyll fluorescence in petioles 9 h after transfer to P5C-containing MS medium (Fig. 7A). Under the same conditions, even 100 mM Pro only weakly affected whole plants within 3 d (Fig. 7D), indicating that P5C is the effector of Pro-induced cell death (Fig. 7C).

Effects of Pro, Suc, and Sorbitol on Gene Expression in Pat(B33)-*Gus* and *rsr1-1*

Regulation of Pro metabolism is well understood, and corresponding genes involved in Pro biosynthesis and ca-

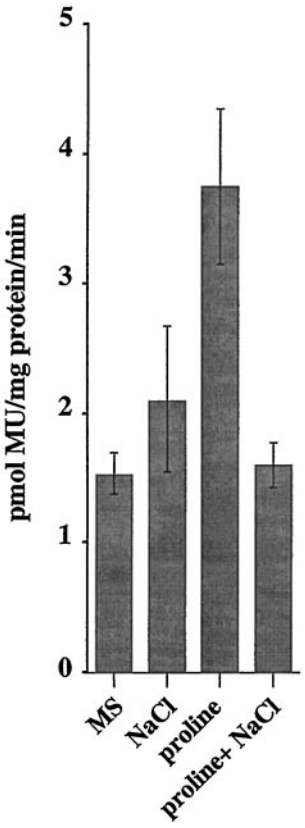

Figure 5. Pro-specific induction of the patatin promoter was blocked by the presence of salt. GUS activity was quantified in roots of 30-d-old plants incubated in liquid MS medium for 30 h in the presence of 200 mM Pro, 100 mM NaCl, or a combination of both.

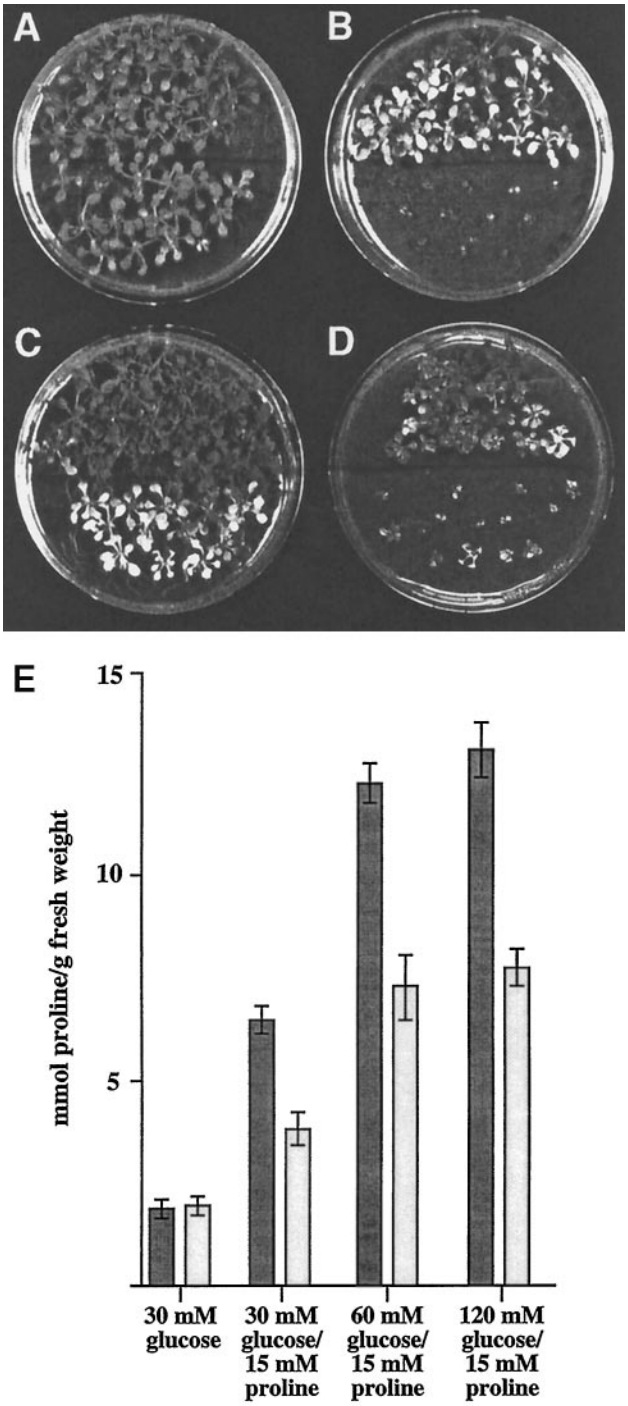

Figure 6. Amelioration of Pro toxicity by Glc but not by nonmetabolizable 3Omeg. Four-week-old Arabidopsis seedlings grown in the presence of 30 mM Glc without (A) or with 15 mM Pro (B). Pat(B33)-*Gus* (upper half of the plates) bleached in the presence of 15 mM Pro, the mutant *rsr1-1* (lower half of the plates) was already dead. Increase of the Glc concentration to 120 mM enabled *rsr1-1* to survive and Pat(B33)-*Gus* to grow normally (C), whereas a combination of 30 mM Glc and 90 mM 3Omeg was ineffective (D). E, Internal Pro content positively correlated with increasing external Glc concentration. Pro increase was more pronounced in Pat(B33)-*Gus* (dark-shaded bars) than in *rsr1-1* (light-shaded bars).

tabolism have been cloned (Verbruggen et al., 1993; Kiyosue et al., 1996). The mutation in *AtRSR1* might lead to altered Pro or P5C-mediated signaling or to altered imino acid metabolism. Therefore, gene expression of *P5CS*, *ProDH*, and sugar-responsive genes was tested under various conditions in mutant and wild-type plants. To obtain better growth, plants were pre-cultured on 2MS medium (60 mM Suc) for 30 d, and were then transferred for 8 or 24 h to liquid MS medium supplemented with Pro, Suc, or sorbitol as an osmotic control. Both the mutant and Pat(B33)-*Gus* responded to Pro and carbohydrates (Fig. 8). *ProDH* expression was induced by Pro and repressed by sorbitol and Suc. Interestingly, *rsr1-1* exhibited a stronger induction of ProDH expression after treatment with Pro or MS medium (Fig. 8, lanes 4, 5, 11, and 12). Pat(B33)-*Gus* reached comparably high transcript levels in the root after 8 h (lanes 1 and 2), but after 24 h expression decreased (lanes 7 and 8).

Both Pat(B33)-*Gus* and *rsr1-1* showed a clear overall reduction of *ProDH* expression upon treatment with sorbitol (lanes 10 and 14) and even lower transcript levels after treatment with Suc (lanes 9 and 13). In roots of *rsr1-1* residual expression of *ProDH* was always detected. These findings indicate that hypersensitivity of *rsr1-1* is due to increased degradation of Pro. After incubation in 100 mM Glc or 3Omeg for 24 h, expression of *ProDH* was reduced in both *rsr1-1* and Pat(B33)-*Gus* (data not shown). In contrast to reduction of *ProDH* expression, 3Omeg was less potent than Glc to ameliorate Pro toxicity under permanent culture conditions, indicating that amelioration depends on

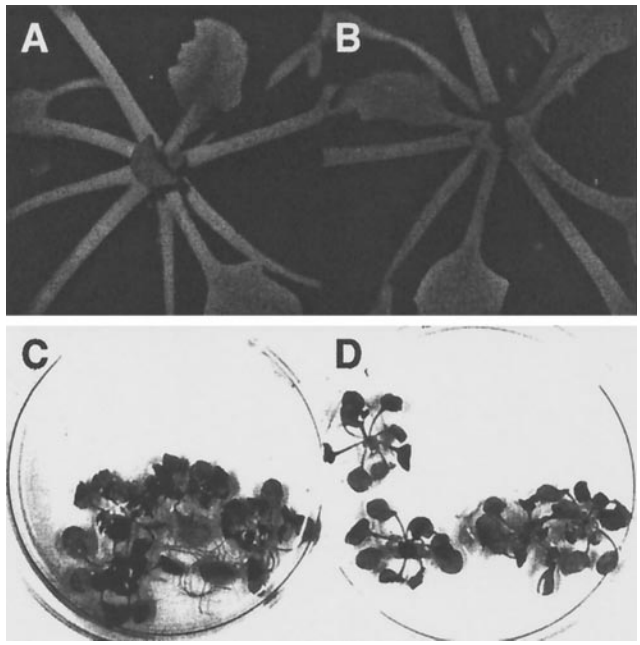

Figure 7. P5C induces Pro-like damages in Arabidopsis. A, Reduction of chlorophyll fluorescence of P5C-treated Pat(B33)-*Gus* plant (1 mM for 9 h) and B, P5C-untreated Pat(B33)-*Gus* plant. Plants treated for 3 d with 1 mM P5C showed brown dead tissue (C), whereas after treatment with 100 mM Pro only minor damage appeared (D). The yellow color in C derives from 2,4-dinitrophenylhydrazine hydrochloride double salt of P5C.

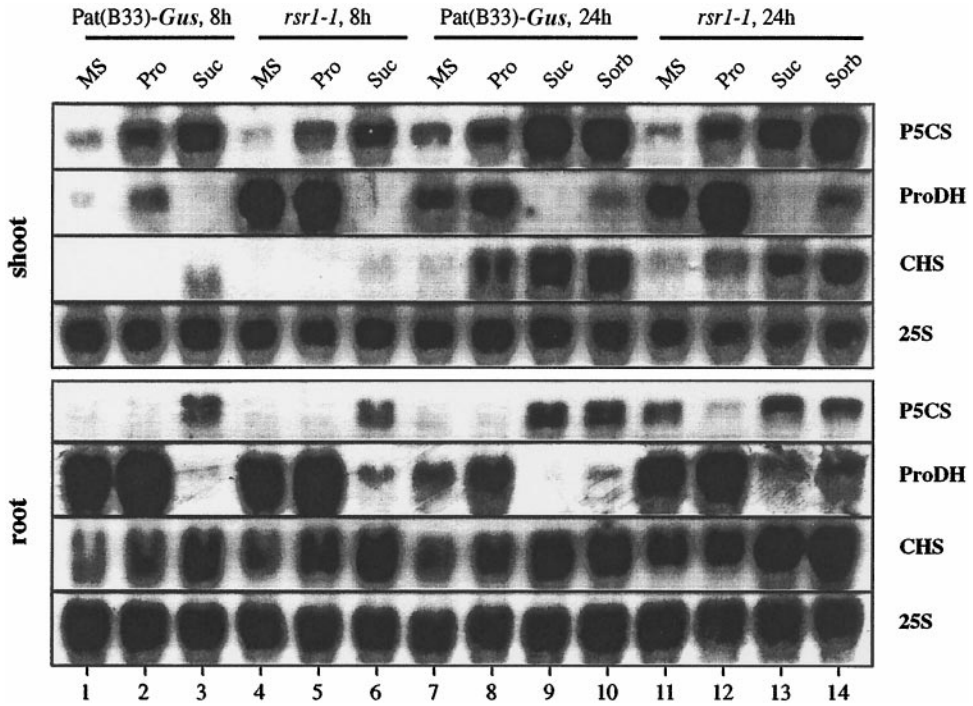

Figure 8. RNA gel-blot analysis of the expression of the *ProDH*, *P5CS*, and *CHS* genes in 3-week-old non-bolting Pat(B33)-*Gus* and *rsr1-1* plants after various treatments. Plants were pre-cultured on 2MS medium for 30 d, and transferred to liquid MS medium containing 200 mM Pro, Suc, or sorbitol, respectively. Root and leaf material was harvested after 8 and 24 h of incubation. Fifteen micrograms of total RNA was loaded in each lane.

metabolization of imported carbohydrates. *P5CS* gene expression is regulated inversely to *ProDH* in both Pat(B33)-*Gus* and the mutant, whereas chalcone synthase (*CHS*) expression was induced by sorbitol and Suc, but only weakly by Pro. All three analyzed genes respond differentially to supply of Pro, Suc, and osmotic stress. In addition, inhibition of *ProDH* expression by Suc was in general stronger than that observed for sorbitol, giving evidence for the presence of carbohydrate-specific effects in addition to osmotic effects.

Genetic Approach to Unravel P5C Toxicity

Increased expression of *ProDH* might lead to elevated P5C levels within the plant if P5C dehydrogenase (P5CDH), the enzyme converting P5C to Glu, is not activated proportionately. Due to its instability, P5C is difficult to measure. Thus, it is important to determine whether the P5CDH gene is also up-regulated. However, so far, the respective gene has not been identified from plants and no plant homologs to mammalian or yeast P5CDH genes can be found in the GenBank (as of September 21, 1999). At least two possibilities remain to prove whether P5C is responsible for the toxic effects of Pro: (a) Measurement of activities of ProDH and P5CDH, or (b) identification of further Pro-hypersensitive mutants that might be affected in P5CDH activity. Therefore, EMS-mutagenized seeds were used (Martin et al., 1997) to screen for Pro-hypersensitive plants and several putative mutants were identified. One of these mutants, *pro*^{HS}*2-1*, was characterized further. The mutation is recessive, as shown by complementation in the F_1 generation of a backcross to the parental line Pat(B33)-*Gus*. Crosses with *rsr1-1* demonstrated that *pro*^{HS}*2-1* is a second and independent locus for Pro hypersensitivity (Fig. 9). Further analyses will show whether *pro*^{HS}*2-1* is mutated in a second regulatory step of the signaling pathway or in the Pro degradation pathway.

DISCUSSION

Regulation of the Patatin Promoter in Arabidopsis

Arabidopsis and potato are only distantly related species, but sink-specific sugar-dependent regulation of the patatin class I promoter is conserved in both species. We have shown that the imino acid Pro, which induces the activation of sugar-responsive elements of the patatin class I promoter in potato (Grierson et al., 1994; K. Beggs and M. Bevan, unpublished data), is also a potent inducer in Arabidopsis. Regulation of the patatin promoter is a new example for metabolite-dependent mechanisms of gene regulation that might be highly conserved in plants. The inhibitory effect of okadaic acid on patatin promoter activity indicates the presence of a signaling cascade that includes protein phosphatases. Since *rsr1-1* was originally identified as a mutant affected in sugar signaling, the additional defect in Pro-dependent induction of the patatin promoter characterizes AtRSR1 as a part of a central signaling pathway connecting C and N metabolism.

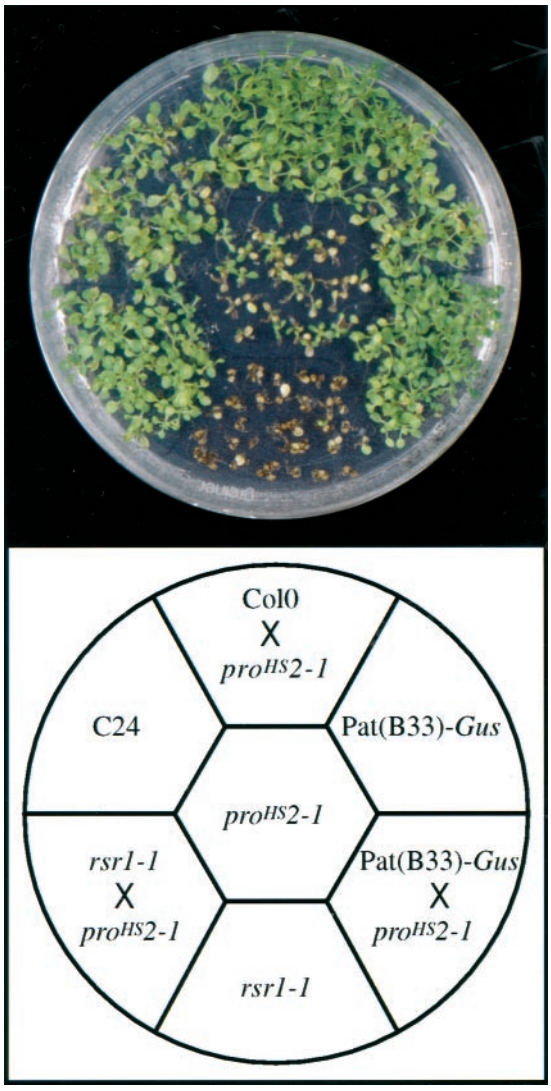

Figure 9. Genetic analysis of *pro*^{HS}*2-1*, a second Pro-hypersensitive mutant. Homozygous *pro*^{HS}*2-1* plants (center) were crossed to Pat(B33)-*Gus*, wild-type Arabidopsis ecotype Col0 and *rsr1-1*. F_1 progeny and parental lines were cultured on MS medium supplemented with 30 mM Glc and 20 mM Pro. Pro hypersensitivity of *pro*^{HS}*2-1* was complemented in all crosses.

It is, however, unlikely that Pro itself is the signal activating the patatin promoter. Under salt-stress conditions, when Pro was shown to accumulate to high concentrations in the plant (Büssis and Heineke, 1998), Pro-dependent induction of the patatin promoter was reduced. Since salt stress negatively affects both Pro-dependent induction of *ProDH* expression and patatin promoter activity (Hare et al., 1999), it is more likely that steps in Pro catabolism are required for activation of the patatin promoter. However, it cannot be excluded that NaCl acts independently on both Pro degradation and regulation of the patatin promoter. P5C might be the activating intermediate, although it did not induce GUS expression in roots under the chosen conditions. This could be due to the low concentrations tested (the form of P5C we used is poorly soluble), rapid cellular degradation, or low uptake rates. Alternatively, ProDH might be involved in regulating the patatin promoter.

Toxicity of Pro Is Caused by Catabolic Steps and Accumulation of Intermediates

The observation of Pro toxicity under non-stressed growth conditions is surprising. Many plant species accumulate high cytosolic concentrations of Pro under abiotic stress conditions such as salt, drought, or cold stress. Furthermore, Pro serves as a compatible solute to protect macromolecular structures, as a radical scavenger, or as a rapid source of energy for recovery from stress (Hare and Cress, 1997; Nanjo et al., 1999). Therefore, it is very unlikely that the observed toxicity is caused by the imino acid itself but rather by Pro-specific catabolic processes.

Amelioration of Pro toxicity in the presence of salt and Glc further strengthened the hypothesis that Pro degradation is required for toxicity. Both treatments reduce transcript levels of *ProDH* and increase Pro content (Kiyosue et al., 1996). In contrast to Glc, the non-metabolizable Glc analog 3Omeg is not able to ameliorate Pro toxicity, demonstrating that Glc metabolism is required for amelioration. Thus, the effect of high Glc concentrations cannot simply be a matter of increasing osmolarity in the medium.

Degradation of Pro leads to the production of reduction equivalents, P5C, and Glu. It has been demonstrated that Glu is not toxic and GABA treatment provides indirect evidence that overproduction of reduction equivalents is also not responsible for Pro-induced damages. Degradation of GABA resembles Pro catabolism in producing Glu and reduction equivalents (Tuin and Shelp, 1996). Even 150 mM GABA did not mimic the toxic effects of 15 mM Pro, making a simple overflow of reduction equivalents rather unlikely. However, in the case of Pro degradation, the actual electron acceptor is still unknown and it remains possible that Pro toxicity is at least in part caused by changes of reduction equivalent levels. So far, the only intermediate proven to be toxic is P5C. Compared with Pro, visible damages appear already at very low concentrations and within relatively short incubation times (1 mM after 3 d), strongly suggesting that P5C is the trigger of Pro-induced toxicity.

P5C is chemically unstable (Mezl and Knox, 1976). Therefore, the determination of P5C in plant material is problematic. Thus, a genetic approach to identify genes involved in Pro toxicity and Pro signal transduction was chosen. Detailed analyses of the identified Pro-hypersensitive mutants and cloning of the respective genes will provide further insight into the mechanism of Pro toxicity.

The concomitant appearance of autofluorescence and necrotic tissue caused by Pro treatment resembles production of phenolic compounds and lignification as a response to pathogen infection. These processes have been described as indicators of apoptosis (Freytag et al., 1994; Ryerson and Heath, 1996). In addition, Iyer and Caplan (1998) reported the induction of stress-related genes in rice by P5C, but did not clarify whether this induction was due to P5C being toxic. Thus, the observed Pro-dependent damage can be caused by accumulating toxic P5C. Cyclic P5C is in equi-

librium with Glu semialdehyde and is unstable in aqueous solution (Mezl and Knox, 1976). Since aldehydes are highly reactive, Glu semialdehyde might react with various cellular compounds and thereby develop toxicity. Alternatively, P5C might act as a signal-molecule activating processes related to apoptosis. It remains to be investigated whether P5C is taken up and sensed by the plant or is simply toxic both intra- and extracellularly.

AtRSR1 Is a Negative Effector of ProDH Gene Expression

The observed amelioration of Pro toxicity by carbohydrates and NaCl is reflected by changes in the expression of genes involved in Pro metabolism and stress response. In addition, the up-regulation of *ProDH* expression by Pro and down-regulation by carbohydrates supports the thesis that Pro degradation is necessary for toxicity. Exogenously supplied Pro is taken up by endogenous transport systems and will lead to increased cellular Pro levels (Rentsch et al., 1996; Fischer et al., 1998). Under these conditions the increased *ProDH* expression will result in a high turnover of imported Pro and probably accumulation of toxic intermediates. The derepression of *ProDH* gene expression in *rsr1-1* indicates that the mutant degrades Pro faster than the Arabidopsis wild type. This would also explain the Pro hypersensitivity of the mutant.

Nakashima et al. (1998) reported that Suc-dependent repression of *ProDH* is due to osmotic effects. However, treatment with either 200 mM Suc or sorbitol indicated a distinct effect of Suc, since sorbitol was slightly less potent at repressing *ProDH* gene expression in both Pat(B33)-*Gus* and *rsr1-1*. However, these treatments are not directly comparable because Suc is taken up via specific transport proteins (Lalonde et al., 1999), whereas no uptake system for sorbitol is known in Arabidopsis. Thus, the difference between extra- and intracellular osmotic potential after 24 h of incubation time is probably less for Suc than for sorbitol. This supports the possibility of Suc-specific regulation in addition to osmotic effects. Salt treatment also reduces transcript levels of *ProDH* (Zhang et al., 1997). Since both Glc and NaCl ameliorate Pro toxicity, the most probable reason for amelioration is a reduced Pro catabolism by repression of *ProDH* expression. This corresponds to elevated Pro content in dependence on increasing concentrations of exogenously supplied Glc. Sorbitol and 3Omeg also have a negative effect on *ProDH* expression, but compared with Glc, both are less potent in reducing Pro toxicity when plants are permanently cultured on Pro-containing medium. This strongly argues for the presence of two different mechanisms regulating *ProDH* expression: A short-term osmotic effect of both sugar and sorbitol and an additional long-term, sugar-specific effect that is dependent on metabolic processes of imported carbohydrates.

The simplest explanation for the results presented assumes that AtRSR1 is part of a signaling cascade triggered by Pro degradation and leading to induction of the patatin promoter but reducing *ProDH* expression. This would provide a feedback mechanism that protects the plant against toxic effects of Pro degradation during recovery from water stress. The regulation of the patatin promoter by both carbohydrates and Pro in combination with the reports on crosstalk between ethylene, cytokinin, and Glc signal transduction (Martin et al., 1997; Zhou et al., 1998; Roitsch, 1999), provide strong evidence for the presence of a complex network connecting various regulatory pathways and carbohydrate metabolism.

MODEL AND CONCLUSIONS

The accumulation of Pro is essential for plants in periods of osmotic stress (Nanjo et al., 1999). However, during recovery, toxicity of Pro degradation products requires an accurate regulation of this process. The signaling pathway including AtRSR1 performs a double function: On the one hand, Pro degradation serves as a signal for stress recovery and allows the induction of the patatin promoter and endogenous genes, which is mediated by AtRSR1. On the other hand, AtRSR1 simultaneously acts as a negative regulator of *ProDH* expression and thereby prevents toxicity of excess Pro degradation (Fig. 10). The mutation in *rsr1-1* disrupts both functions. The plant is no longer able to

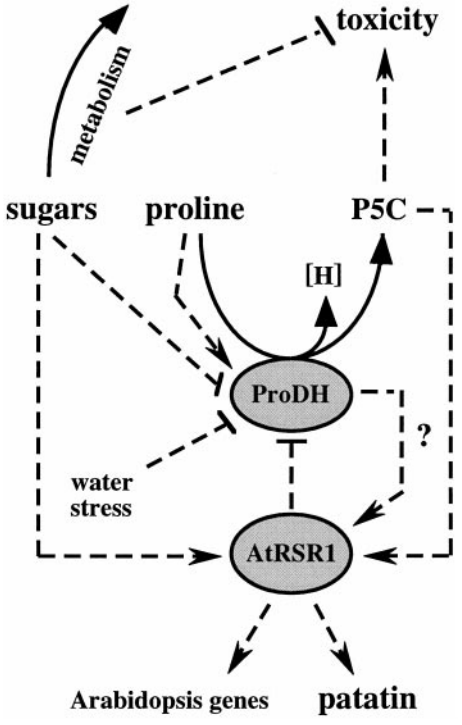

Figure 10. Working model for the position of AtRSR1 in the regulatory network connecting Pro and carbohydrate signaling with the patatin promoter. AtRSR1 is induced by sugars and Pro degradation, but in the latter case the actual effector remains unclear. Activation of AtRSR1 leads to repression of *ProDH* expression and induces the patatin promoter. Endogenous target genes other than *ProDH* remain to be identified. Toxicity of Pro derives from the overproduction of P5C in the absence of stress. The mutation in *AtRSR1* abolishes feedback inhibition of Pro degradation and thereby produces hypersensitivity toward Pro. Amelioration of toxicity can be achieved by hyperosmolar conditions that reduce *ProDH* transcript levels or by supply of metabolizable carbohydrates that potentially interfere on a different level.

induce the patatin class I promoter and becomes hypersensitive to Pro. Since P5C, under the chosen conditions, was not able to induce patatin promoter activity, toxicity and the regulation of the patatin promoter are probably independent processes. Regulation of the patatin promoter in Arabidopsis might be dependent on ProDH, so further investigation of ProDH in relation to patatin promoter activity will be of high importance.

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LITERATURE CITED

Ahmad I, Hellebust JA (1988) The relationship between inorganic nitrogen metabolism and proline accumulation in osmoregulatory responses of two eurythaline microalgae. Plant Physiol **88:** 348–354

Balibrea ME, Rus-Alvarez AM, Bolarin MC, Perez-Alfocea F (1997) Fast changes in soluble carbohydrates and proline contents in tomato seedlings in response to ionic and non-ionic iso-osmotic stresses. J Plant Physiol **151:** 221–226

Bates LS, Waldren RP, Teare ID (1973) A rapid determination of free proline for water-stress studies. Plant Soil **39:** 205–207

Blum A, Ebercon A (1976) Genotypic responses in sorghum to drought stress. III. Free proline accumulation and drought resistance. Crop Sci **16:** 428–431

Büssis D, Heineke D (1998) Acclimation of potato plants to polyethylene glycol-induced water deficit. II. Contents and subcellular distribution of organic solutes. J Exp Bot **49:** 1361–1370

Chen DM, Keiper FJ, De Filippis LF (1998) Physiological changes accompanying the induction of salt tolerance in *Eucalyptus microcorys* shoots in tissue culture. J Plant Physiol **152:** 555–563

Clifford SC, Arndt SK, Corlett JE, Joshi S, Sankhla N, Popp M, Jones HG (1998) The role of solute accumulation, osmotic adjustment and changes in cell wall elasticity in drought tolerance in *Ziziphus mauritiana* (Lamk.). J Exp Bot **49:** 967–977

Fischer WN, André B, Rentsch D, Krolkiewicz S, Tegeder M, Frommer WB (1998) Amino acid transport in plants. Trends Plant Sci **3:** 188–195

Freytag S, Arabatzis N, Halbrock K, Schmelzer E (1994) Reversible cytoplasmic rearrangements wall apposition, hypersensitive cell death and defense-related gene activation in potato/*Phytophtora infestans* interactions. Planta **194:** 123–135

Grierson C, Du JS, Zabala MDT, Beggs K, Smith C, Holdsworth M, Bevan M (1994) Separate cis sequences and trans factors direct metabolic and developmental regulation of a potato tuber storage protein gene. Plant J **5:** 815–826

Hare PD, Cress WA (1997) Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regul **21:** 79–102

Hare PD, Cress WA, van Staden J (1999) Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. J Exp Bot **50:** 413–434

Heineke D, Sonnewald U, Büssis D, Günter G, Leidreiter K, Wilke I, Raschke K, Willmitzer L, Heldt HW (1992) Apoplastic expression of yeast-derived invertase in potato. Plant Physiol **100:** 301–308

Iyer S, Caplan A (1998) Products of proline catabolism can induce osmotically regulated genes in rice. Plant Physiol **116:** 203–211

Kiyosue T, Yoshiba Y, Yamaguchi-Shinozaki K, Shinozaki K (1996) A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in *A. thaliana*. Plant Cell **8:** 1323–1335

Lalonde S, Boles E, Hellmann H, Barker L, Patrick JW, Frommer WB, Ward JM (1999) The dual function of sugar carriers: transport and sugar sensing. Plant Cell **11:** 707–726

Lam HM, Peng SSY, Coruzzi GM (1994) Metabolic regulation of the gene encoding glutamine-dependent asparagine synthetase in *Arabidopsis thaliana*. Plant Physiol **106:** 1347–1357

Larher F, Leport L, Petrivalsky M, Cappart M (1993) Effectors for the osmoinduced proline response in higher plants. Plant Physiol Biochem **31:** 911–922

Lehrach H, Diamon D, Wozney JM, Boedtker H (1977) RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16:** 4743–4751

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem **163:** 16–20

Martin T, Hellmann H, Schmidt R, Willmitzer L, Frommer WB (1997) Identification of mutants in metabolically regulated gene expression. Plant J **11:** 53–62

Mezl VA, Knox WE (1976) Properties and analysis of a stable derivative of pyrroline-5-carboxylic acid for use in metabolic studies. Anal Biochem **74:** 430–440

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Nakashima K, Satoh R, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1998) A gene encoding proline dehydrogenase is not only induced by proline and hypoosmolarity, but is also developmentally regulated in the reproductive organs of Arabidopsis. Plant Physiol **118:** 1233–1241

Nanjo T, Kobayashi M, Yoshiba Y, Sanada Y, Wada K, Tsukaya H, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (1999) Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. Plant J **18:** 185–193

Nielsen TH, Krapp A, Roeper-Schwarz U, Stitt M (1998) The sugar-mediated regulation of genes encoding the small subunit of Rubisco and the regulatory subunit of ADP glucose pyrophosphorylase is modified by phosphate and nitrogen. Plant Cell Environ **21:** 443–454

Peng Z, Lu Q, Verma DPS (1996) Reciprocal regulation of DELTA-1-pyrroline-5-carboxylate synthetase and proline dehydrogenase genes controls proline levels during and after osmotic stress in plants. Mol Gen Genet **253:** 334–341

Pesci P (1993) Glucose mimics the enhancing effect of light on ABA-induced proline accumulation in hydrated barley and wheat leaves. J Plant Physiol **142:** 355–359

Rentsch D, Hirner B, Schmelzer E, Frommer WB (1996) Salt stress-induced proline transporters and salt stress-repressed broad specific amino acid permeases identified by suppression of a yeast amino acid targeting mutant. Plant Cell **8:** 1437–1446

Rhodes D, Handa S, Bressan RA (1986) Metabolic changes associated with adaptation of plant cells to water stress. Plant Physiol **82:** 890–903

Rocha-Sosa M, Sonnewald U, Frommer WB, Stratmann M, Schell J, Willmitzer L (1989) Both developmental and metabolic signals activate the promoter of a patatin class I gene. EMBO J **8:** 23–29

Roitsch T (1999) Source-sink regulation by sugar and stress. Curr Op Plant Biol **2:** 198–206

Ryerson DE, Heath MC (1996) Cleavage of nuclear DNA into oligonucleosomal fragments during cell death induced by fungal infection or by abiotic treatments. Plant Cell **8:** 393–402

Savoure A, Jaoua S, Hua XJ, Ardiles W, Van Montagu M, Verbruggen N (1995) Isolation, characterization, and chromosomal location of a gene encoding the DELTA-1-pyrroline-5-carboxylate synthetase in *Arabidopsis thaliana*. FEBS Lett **372:** 13–19

Schobert B, Tschesche H (1978) Unusual solution properties of proline and its interaction with proteins. Biochim Biophys Acta **541:** 270–277

Schwacke R, Grallath S, Breitkreuz KE, Stransky E, Stransky H, Frommer WB, Rentsch D (1999) LeProT1, a transporter for proline, glycine-betaine and γ-aminobutyric acid in tomato pollen. Plant Cell **11:** 377–391

Smirnoff N, Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry **28:** 1057–1060

Takeda S, Mano S, Ohto M, Nakamura N (1994) Inhibitors of protein phosphatases 1 and 2A block the sugar-inducible gene expression in plants. Plant Physiol **106:** 567–574

Tuin LG, Shelp BJ (1996) In situ ^{14}C-glutamate metabolism by developing soybean cotyledons. II. The importance of glutamate decarboxylation. J Plant Physiol **147:** 714–720

Verbruggen N, Villarroel R, Van Montagu M (1993) Osmoregulation of a pyrroline-5-carboxylate reductase gene in *Arabidopsis thaliana*. Plant Physiol **103:** 771–781

Voetberg GS, Sharp RE (1991) Growth at the maize primary root at low water potential. III. Role of increased proline deposition in osmotic adjustment. Plant Physiol **96:** 1125–1130

Wyn Jones RG, Storey R, Leigh RA, Ahmad N, Pollard A (1977) A hypothesis on cytoplasmic osmoregulation. *In* E Marr, O Ciferri, eds, Regulation of Cell Membrane Activities in Plants. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 121–136

Zhang CS, Lu Q, Verma DPS (1997) Characterization of DELTA-1-pyrroline-5-carboxylate synthetase gene promoter in transgenic *Arabidopsis thaliana* subjected to water stress. Plant Sci **129:** 81–89

Zhou L, Jang JC, Jones TL, Sheen J (1998) Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. Proc Natl Acad Sci USA **95:** 10294–10299