Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*

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Abstract Synthesis, degradation, and transport of proline (Pro) are thought to cooperatively control its endogenous levels in higher plants in response to environmental conditions. To evaluate the function of Pro degradation in the regulation of the levels of Pro and to elucidate roles of Pro in stress tolerance, we generated antisense transgenic *Arabidopsis* plants with an AtProDH cDNA encoding proline dehydrogenase (ProDH), which catalyzes Pro degradation. Several transgenic lines accumulated Pro at higher levels than wild-type plants, providing evidence for a key role of ProDH in Pro degradation in *Arabidopsis*. These antisense transgenics were more tolerant to freezing and high salinity than wild-type plants, showing a positive correlation between Pro accumulation and stress tolerance in plants.

Key words: Antisense; Proline dehydrogenase; Freezing tolerance; Salinity tolerance; Hydroponic culture

1. Introduction

Many plants accumulate free proline (Pro) in response to the imposition of environmental stresses such as drought, high salinity, and low temperature [1]. Under stressed conditions, Pro acts as a mediator of osmotic adjustment [2], a stabilizer of subcellular structures [3], a scavenger of free radicals [4], a buffer in cellular redox potential [1], and a major constituent of cell wall structural proteins that may provide mechanical support for cells [5]. Thus, Pro is regarded as having multiple roles in stress tolerance in plants. Many reports have indicated a positive correlation between the accumulation of Pro and stress tolerance in plants [6,7]. Strong evidence for the relationship between Pro levels and osmotolerance was obtained based on salt tolerance in transgenic tobacco plants with enhanced levels of Pro biosynthesis [6]. However, other reports have proposed that an increased free Pro level is merely a result of stress [8]. Thus, the roles of Pro in osmotolerance in plants remain controversial, and more studies on the function of Pro in stress tolerance are necessary.

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Abbreviations: Pro, proline; P5C, Δ^1-pyrroline-5-carboxylate; GSA, glutamic-γ-semialdehyde; P5CS, P5C synthase; P5CR, P5C reductase; ProDH, proline dehydrogenase; P5CDH, P5C dehydrogenase

In higher plants, Pro is synthesized mainly via the glutamic acid (Glu) pathway [6,9]. Pro accumulation in stressed plants is caused by both the activation of Pro biosynthesis and the inactivation of Pro degradation [10]. Pro is synthesized from Glu via two intermediates, glutamic-γ-semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate (P5C). Two enzymes catalyze this pathway, P5C synthase (P5CS) in the first step and P5C reductase (P5CR) in the final step (Fig. 1A). Genes encoding enzymes in Pro biosynthesis have been isolated from various plants, and their expression and the functions of their products have been characterized [11]. On the other hand, Pro is metabolized to Glu via P5C and GSA. Two enzymes catalyze this pathway, proline dehydrogenase (ProDH) in the first step and P5C dehydrogenase (P5CDH) in the final step (Fig. 1A). A drought-inducible gene, *ERD5*, defined as *AtProDH* in this paper, encoding a precursor of ProDH has been isolated from *Arabidopsis* [10]. The same gene was reported by two other groups [12,13]. Expression of the *AtProDH* gene is induced by both the application of exogenous Pro and rehydration, but is repressed by dehydration [10,11].

To investigate the function of Pro degradation in the accumulation of Pro and to further elucidate roles of Pro in stress tolerance in plants, we generated antisense transgenic *Arabidopsis* plants with an AtProDH cDNA. Several transgenics showed enhanced accumulation of Pro, providing evidence for a pivotal role of ProDH in Pro degradation. These transgenic plants showed tolerance to freezing and high salinity. We showed the efficiency of suppressing Pro degradation in Pro accumulation, and the contribution of Pro to stress tolerance in higher plants.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia was used in this study. Plants were grown on GM agar plates [14] as described by Nanjo et al. [5] unless otherwise mentioned. One-week-old seedlings were transferred to a new GM plate and then grown for another week. Two-week-old plate-grown plants were transferred to 64 mm diameter stainless steel tea strainers filled with 150 g of quartz sand for hydroponic culture with one-fifth strength Hoagland's nutrient solution [15], or were transferred to 9-cm plastic pots filled with perlite.

2.2. Construction of transgenic plants

An AtProDH (ERD5) cDNA [10] encoding *Arabidopsis* ProDH protein was reverse-fused to the *Bam*HI site of the expression vector pBE2113 [16], which contained a 35S cauliflower mosaic virus pro-

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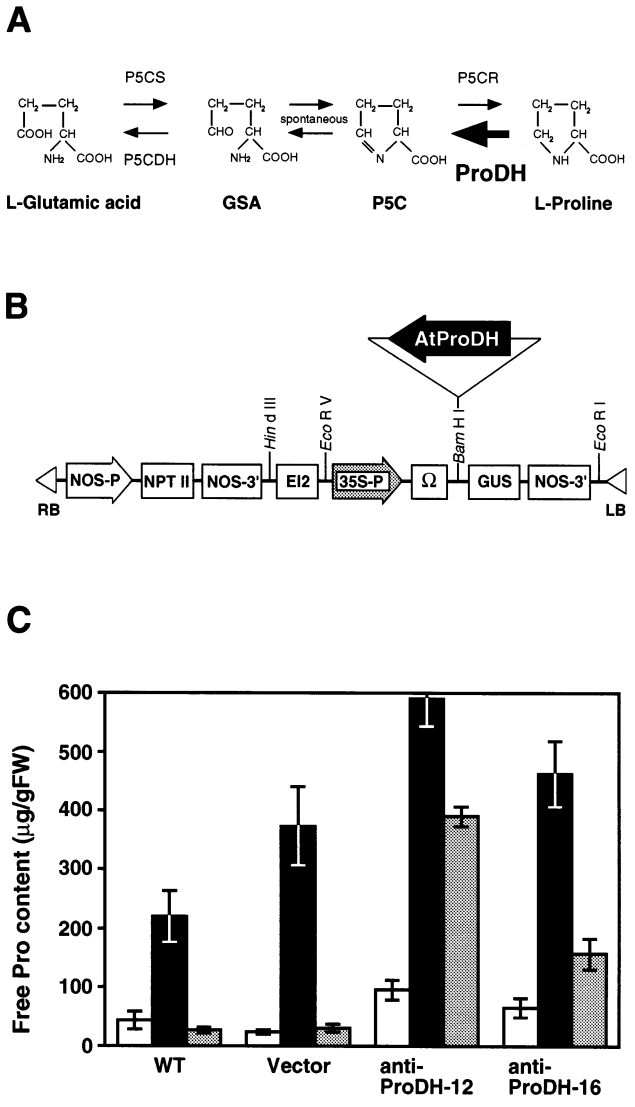

Fig. 1. Establishment of 35S-AtProDH antisense transgenic *Arabidopsis*. A: Metabolic pathway of Pro in plants (the Glu pathway). GSA, glutamic-γ-semialdehyde; P5C, Δ^1-pyrroline-5-carboxylate; P5CS, P5C synthase; P5CR, P5C reductase; ProDH, proline dehydrogenase; P5CDH, P5C dehydrogenase. B: Construction of the 35S-AtProDH antisense DNA fusion gene. An AtProDH cDNA [10] was inserted into the *Bam*HI site of vector pBE2113 [16], and then plasmids containing the AtProDH cDNA in the reverse direction were identified by sequence analysis and used for the transformation of *Arabidopsis*. C: Free Pro contents in 3-week-old rosette leaves from *Arabidopsis* plants grown on GM. Wild-type (WT) plants, transgenics with the empty vector pBE2113 (Vector), and AtProDH antisense transgenics (anti-ProDH-12 and -16) before (white) and after the 48-h low-humidity treatment [5] (black), and after subsequent 17-h rehydration (gray). Kanamycin in the medium did not affect the growth or the mutated phenotypes of the transgenics. Means ± S.E.M. of three independent experiments are shown.

moter (Fig. 1B). The resultant plasmid was introduced into wild-type *Arabidopsis* seedlings, and transgenic plants were finally obtained as described by Nanjo et al. [5]. Among the T_2 plants, lines that were homozygous with respect to the introduced genes were selected by examining the distribution of kanamycin resistance in their seeds (T_3 seeds) after self-pollination. T_3 seeds were used for subsequent experiments.

2.3. RNA gel blot analysis

Total RNA was isolated from rosette leaves of plants that had been grown on GM plates for 3 weeks and then subjected to 260 mM L-Pro for 10 h. Twenty micrograms of total RNA was fractionated by electrophoresis on a 1% agarose gel containing formaldehyde and blotted onto a nitrocellulose filter. The membrane was probed with ^{32}P-labeled AtProDH antisense RNA, which detects ~1.8 kbp of AtProDH mRNA. The AtProDH antisense RNA probe was used for Northern hybridization. Isolation and fractionation of total RNA and the hybridization were performed as described by Nanjo et al. [5].

2.4. Freezing and salinity stress treatment

Freezing stress was imposed by exposing 3-week-old soil-grown plants to −7°C under continuous illumination for 2 days. After the freezing treatment, the plants were returned to room temperature at 22°C for 5 days, and the survival rates of 50 plants and membrane ion leakage in five independent plants were measured.

Salinity stress was imposed by exposing 4-week-old hydroponically grown plants, which had been chosen for length of a primary inflorescence in the range 50–120 mm, to nutrient medium containing 600 mM NaCl, then returning them to medium without NaCl. Just before the NaCl treatment, the tea strainers holding the plants were put on paper towels for 20 min to drain the sand bed and then transferred to the NaCl solution. We created a new system by combining sand culture with hydroponic culture. We used a hydroponic culture because of the uniformity of growth conditions and stress intensity. Time until lodging of a primary inflorescence was compared (Fig. 4A). We made sure in advance that the height of inflorescences was not related to how long they could withstand the treatment.

2.5. Measurement of membrane leakage

Membrane leakage was determined by measurement of electrolyte leaked from leaves. Six leaves from each treatment group were immersed in 5 ml of 0.4 M mannitol at room temperature with gentle shaking for 3 h, and the solution was measured for conductivity with a conductivity meter (DS-15, Horiba, Kyoto, Japan). The total conductivity was determined after the sample was boiled for 10 min. The conductivity due to leakage is expressed as the percentage of the initial conductivity over the total conductivity.

2.6. Free proline determination

Three-week-old rosette leaves were ground in distilled water. The homogenate was boiled for 6 min and then centrifuged at 10 000 × *g* for 15 min at 4°C. The supernatant was precipitated with 10% tri-

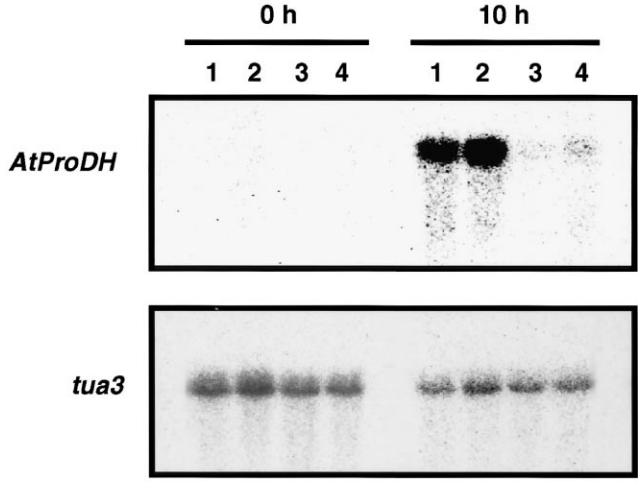

Fig. 2. Repression of the AtProDH mRNA in the AtProDH antisense transgenic plants. Northern blot of total RNA (20 μg per lane) isolated from plants untreated (0 h) and treated with 260 mM L-Pro for 10 h (10 h). The membranes were probed with ^{32}P-labeled AtProDH antisense RNA (upper panel) and α-tubulin 3 cDNA (*tua3*) as a control (lower panel). Plants used for the analysis were wild-type (lane 1), transgenics with the empty vector pBE2113 (lane 2), and AtProDH antisense transgenics (anti-ProDH-12, lane 3; anti-ProDH-16, lane 4).

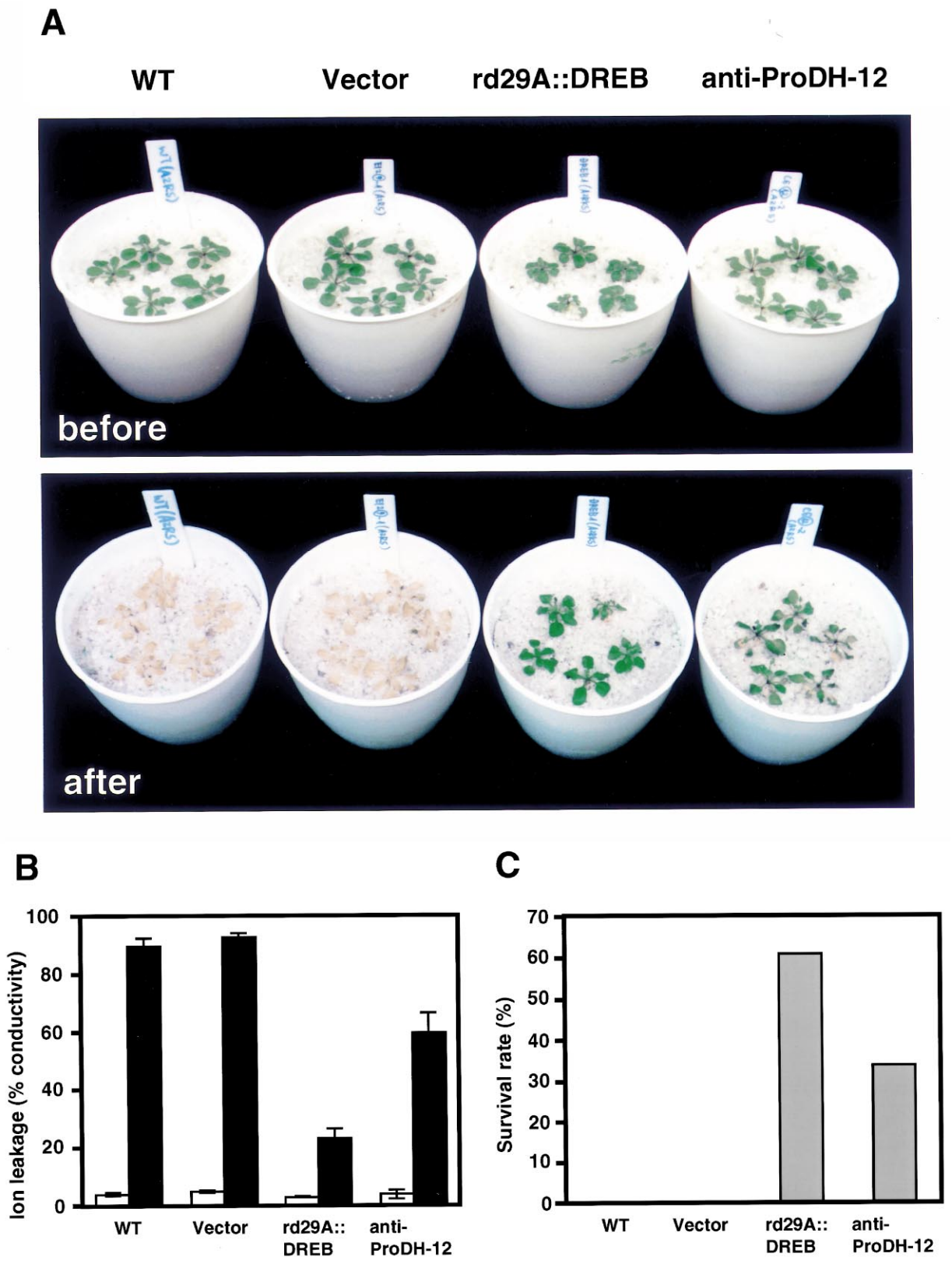

Fig. 3. Freezing tolerance of the AtProDH antisense transgenic plants. A: Phenotype of plants exposed to freezing stress. B: Membrane leakage of plants exposed to freezing stress. Bar heights indicate mean values; error bars represent S.E.M. from three duplicated measurements. C: Survival rate of 50 plants exposed to freezing stress. Plants used for the analysis were wild-type (WT) and transgenics with the empty vector pBE2113 (Vector) as negative controls, rd29A::DREB1A transformant as a positive control, and anti-ProDH-12 plants.

chloroacetic acid (TCA) for 5 h and centrifuged at 10 000×*g* for 20 min. The supernatant after TCA precipitation was derivatized as described by Cohen and Strydom [17], and the contents of free Pro were determined by HPLC (model LC Module I plus, Waters Associates, Milford, MA, USA).

3. Results

3.1. Creation of transgenic lines with an AtProDH cDNA

Forty independent transgenic lines with the AtProDH antisense cDNA were established, and T_3 progeny (named anti-ProDH) were used for further analysis. When free Pro contents of these transgenic seedlings were measured under unstressed, dehydrated, and rehydrated conditions, several lines were found to accumulate more Pro than wild-type plants, even after 17-h rehydration. A variation of the position effect may account for the difference in Pro accumulation among transgenic lines. We selected two lines, anti-ProDH-12 and -16, for further investigations. These plants accumulated more Pro than wild-type plants in either stressed or unstressed conditions (Fig. 1C).

The effect of transformation with the AtProDH antisense cDNA on the amounts of AtProDH transcripts was evaluated by Northern blot analysis with an AtProDH antisense RNA probe. Although AtProDH mRNA accumulated in wild-type plants and in the vector-transformed control plants that re-

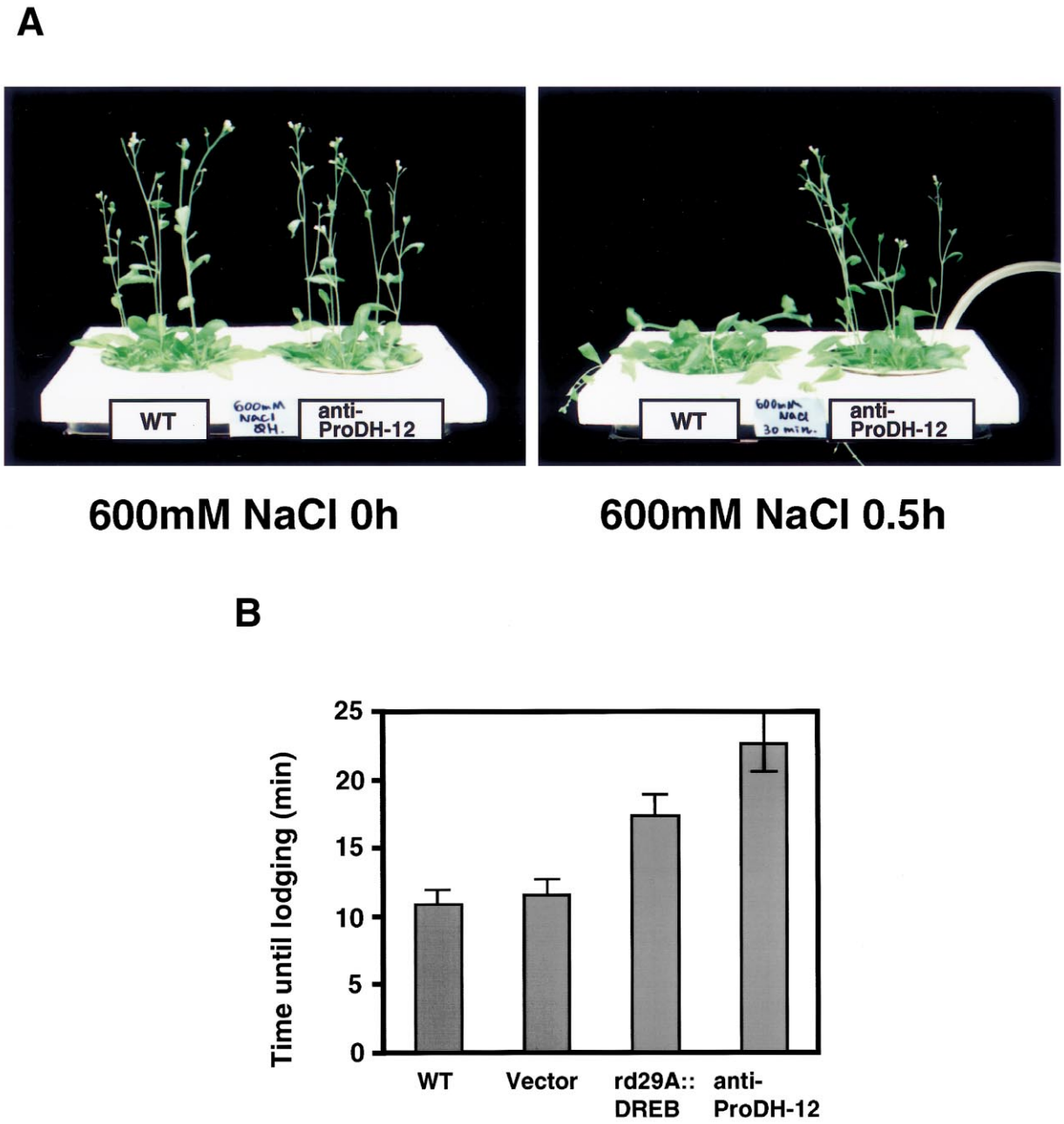

Fig. 4. Salinity tolerance of the AtProDH antisense transgenic plants. A: Phenotype of plants exposed to salinity stress for 0.5 h. B: Time until lodging of a primary inflorescence during 600 mM NaCl treatment. Bar heights indicate mean values; error bars represent S.E.M. from 30 duplicated measurements. Plants used for the analysis were from the same lines as described in the legend of Fig. 3.

sponded to the exogenous L-Pro, it did not accumulate significantly in the anti-ProDH plants (Fig. 2). This confirms the maintenance of free Pro levels after rehydration by the suppression of AtProDH protein synthesis by the antisense transgene.

3.2. Freezing tolerance of anti-ProDH plants

To elucidate the role of Pro in freezing tolerance, we examined the anti-ProDH plants for their tolerance to freezing stress treatment. Although all wild-type and vector-transformed control plants died (Fig. 3A), the anti-ProDH-12 plants showed tolerance to the treatment: 33% survival and 59% ion leakage were observed (Fig. 3B,C). Similar freezing tolerance was observed in the anti-ProDH-16 plants.

As a positive control, we used rd29A::DREB1A transgenic *Arabidopsis*, in which transcription factor DREB1A is driven by the stress-inducible rd29A promoter [18]. It has been reported that rd29A::DREB1A transgenic plants show multiple tolerance to drought, high salinity, and freezing [18]. This positive control also showed freezing tolerance in this study: 60% survival and 23% ion leakage were observed (Fig. 3B,C). The freezing tolerance of the anti-ProDH-12 plants was higher than that of the wild-type and vector-transformed plants, but lower than that of the rd29A::DREB1A transgenic plants.

3.3. Salinity tolerance of anti-ProDH plants

To investigate the role of Pro in salinity tolerance, we examined the anti-ProDH plants for their tolerance to NaCl stress treatment. Although almost all the inflorescences of wild-type and vector-transformed control plants lodged within 12 min, those of the anti-ProDH-12 plants lasted at least 22 min (Fig. 4B). A similar level of tolerance was observed in the anti-ProDH-16 plants. In the rd29A::DREB1A transgenic plants, the inflorescences lodged within 18 min (Fig. 4B). Thus, salinity tolerance of the anti-ProDH plants was highest.

4. Discussion

Many studies have focused on manipulating the accumulation of diverse compatible osmolytes because of their relatively simple biochemical traits and because of current advances in biotechnology, aiming to analyze the osmolytes' biological functions in stress tolerance and to breed stress-tolerant crops. We have focused on Pro, which is one of the most common osmolytes, and studied its potential roles in stress tolerance in plants by transgenic technologies. Stress-inducible accumulation of Pro is caused by both the activation of Pro biosynthesis and the inactivation of Pro degradation [10]. Some authors have dissected the biosynthetic pathway of Pro and have isolated genes involved in the pathway, such as *P5CS* and *P5CR*, from various plant species, proving that P5CS is a key enzyme catalyzing Pro biosynthesis [5,6]. Other authors have focused on the feedback regulation of P5CS and Pro transport that may be involved in Pro accumulation and its use in stress tolerance in plants [9,19,20]. By contrast, the molecular mechanism of Pro degradation is less well understood [11]; for example, a gene encoding P5CDH that converts P5C to Glu [21] has yet to be isolated. In this study, ProDH antisense transgenic *Arabidopsis* (anti-ProDH) plants could maintain Pro accumulation at a high level even after rehydration by the suppression of ProDH. Thus, we have directly shown that ProDH has an intensive role in controlling Pro levels in plants.

Kavi Kishor et al. have reported that elevated Pro biosynthesis levels caused by overexpression of mothbean P5CS in transgenic tobacco plants confer improved tolerance to salt stress [6]. Their results provide clear evidence for a positive correlation between the accumulation of Pro and osmotolerance in plants. However, it is still argued whether Pro accumulation is effective in tolerance to abiotic stresses.

In this study, we examined the tolerance of anti-ProDH plants to freezing and salinity stress. The anti-ProDH-12 plants, which showed constitutive accumulation of Pro, tolerated freezing treatment, and some survived (Fig. 3). This supports a previous report that an *Arabidopsis* mutant named *eskimo* accumulated more Pro than wild-type plants by preventing the induction of the *AtProDH* gene, and was constitutively freezing-tolerant [22]. Our anti-ProDH-12 plants showed tolerance to NaCl treatment as well. The anti-ProDH-12 plants that accumulated Pro under both unstressed and stressed conditions showed the longest time until lodging of a primary inflorescence when exposed to 600 mM NaCl (Fig. 4). This suggests that accumulated Pro contributes to salinity tolerance in *Arabidopsis*. In our experiment, plants were directly transferred from normal conditions to −7°C or to 600 mM NaCl. These quick and intensive treatments should be efficient at obtaining clear differences between transgenics and control plants in stress tolerance, because wild-type plants accumulate Pro in response to stress treatments when the treatments are imposed slowly and moderately. We were interested in whether Pro-overaccumulating plants are broadly tolerant to various stresses or especially tolerant to salt stress. Although the anti-ProDH plants showed tolerance to both freezing and salt stresses, their tolerance to salinity was significantly high: the freezing tolerance of the anti-ProDH plants was lower than that of rd29A::DREB1A plants but their salt tolerance was higher than that of this positive control (Figs. 3 and 4). This suggests that over-accumulated Pro in anti-ProDH plants confers a specific resistance to damage due to salt stress.

In conclusion, this is the first report of the efficient accumulation of Pro by the suppression of Pro degradation in antisense ProDH transgenics. We have confirmed the contribution of accumulated Pro to stress tolerance in higher plants. These results will help us improve crop tolerance to abiotic stresses. The constitutive accumulation of Pro due to the inhibition of its degradation may be a promising approach to creating salt-tolerant crops.

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