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Expression of a Late Embryogenesis Abundant Protein Gene, *HVA1*, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice[1]

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A late embryogenesis abundant (LEA) protein gene, *HVA1*, from barley (*Hordeum vulgare* L.) was introduced into rice suspension cells using the Biolistic-mediated transformation method, and a large number of independent transgenic rice (*Oryza sativa* L.) plants were generated. Expression of the barley *HVA1* gene regulated by the rice actin 1 gene promoter led to high-level, constitutive accumulation of the HVA1 protein in both leaves and roots of transgenic rice plants. Second-generation transgenic rice plants showed significantly increased tolerance to water deficit and salinity. Transgenic rice plants maintained higher growth rates than nontransformed control plants under stress conditions. The increased tolerance was also reflected by delayed development of damage symptoms caused by stress and by improved recovery upon the removal of stress conditions. We also found that the extent of increased stress tolerance correlated with the level of the HVA1 protein accumulated in the transgenic rice plants. Using a transgenic approach, this study provides direct evidence supporting the hypothesis that LEA proteins play an important role in the protection of plants under water- or salt-stress conditions. Thus, LEA genes hold considerable potential for use as molecular tools for genetic crop improvement toward stress tolerance.

Environmental stresses, such as water deficit, increased salinity of soil, and extreme temperature, are major factors limiting plant growth and productivity (Epstein et al., 1980; Yancey et al., 1982). In response to various environmental stresses, plants have developed different physiological and biochemical strategies to adapt or tolerate stress conditions. Accumulation of compatible, low-molecular-weight osmolytes, such as sugar alcohols, special amino acids, and Gly betaine, has been suggested as a major mechanism that may underlie the adaptation or tolerance of plants to osmotic stresses (Greenway and Munns, 1980; Yancey et al., 1982). Recently, several studies have demonstrated that genetic manipulation of the accumulation of low-molecular-weight osmolytes resulted in increased tolerance to water or salt stress in transgenic plants (Tarczynski et al., 1993; Holmstrom et al., 1994; Rathinasabapathi et al., 1994; Saneoka et al., 1995).

In addition to metabolic changes and accumulation of low-molecular-weight protective compounds, a large set of plant genes is transcriptionally activated, which leads to accumulation of new proteins in vegetative tissue of plants under osmotic stress conditions (Skriver and Mundy, 1990; Chandler and Robertson, 1994, and refs. therein). It is generally assumed that stress-induced proteins might play a role in tolerance, but direct evidence is still lacking, and the functions of many stress-responsive genes are unknown.

LEA proteins were first characterized in cotton as a set of proteins that are highly accumulated in the embryos at the late stage of seed development (Dure et al., 1981). Subsequently, many LEA proteins or their genes have been characterized from different plant species (collated by Dure, 1992). Based on their common amino acid sequence domains, LEA proteins were classified into three major groups (Baker et al., 1988; Dure et al., 1989). The regions of homology among group 3 LEA proteins are composed of tandem repeats of an 11-amino acid motif that may form an amphiphilic α-helix structure (Baker et al., 1988; Dure et al., 1989; Dure, 1993). It has been hypothesized, based on the correlation of LEA gene expression with physiological and environmental stresses and the predicted novel structure of the LEA proteins, that LEA protein may play a protective role in plant cells under various stress conditions; moreover, this protective role may be essential for the survival of the plant under extreme stress conditions (Baker et al., 1988; Dure et al., 1989; Skriver and Mundy, 1990; Chandler and Robertson, 1994).

The correlation between LEA gene expression or LEA protein accumulation and stress tolerance in a number of plants provides additional evidence supporting a role of LEA proteins in stress tolerance. For example, in severely dehydrated wheat seedlings, the accumulation of high lev-

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Abbreviations: *Act1*, actin 1 gene; CaMV, cauliflower mosaic virus; LEA, late embryogenesis abundant; MS, Murashige-Skoog; NT, nontransformed or wild-type control; *Pin2*, proteinase inhibitor II gene; R_0, first generation; R_1, second generation.

els of group 3 LEA proteins was correlated with tissue dehydration tolerance (Ried and Walker-Simmons, 1993). Studies of several Indica varieties of rice (*Oryza sativa* L.) showed that the levels of group 2 LEA proteins (also known as dehydrins) and group 3 LEA proteins in roots were significantly higher, or induced by ABA and salt stress, only in salt-tolerant varieties as compared with salt-sensitive varieties (Moons et al., 1995). However, clear experimental evidence supporting the exact functions of LEA proteins is still lacking, and the physiological roles of LEA proteins remain largely unknown.

A barley (*Hordeum vulgare* L.) group 3 LEA protein, HVA1, was previously characterized from barley aleurone. The *HVA1* gene is specifically expressed in the aleurone layers and the embryos during late seed development, correlating with the seed desiccation stage (Hong et al., 1988). Expression of the *HVA1* gene is rapidly induced in young seedlings by ABA and by several stress conditions, including dehydration, salt, and extreme temperature (Hong et al., 1992). The barley *HVA1* gene and the wheat *pMA2005* gene (Curry et al., 1991; Curry and Walker-Simmons, 1993) are highly similar at both the nucleotide level and the predicted amino acid level. These two monocot genes are closely related to the cotton *D-7* gene (Baker et al., 1988) and carrot *Dc3* gene (Seffens et al., 1990), with which they share a similar structural gene organization (Straub et al., 1994). A 26-kD group 3 LEA protein was induced by ABA and salt stress only in a salt-tolerant Indica rice variety (Moons et al., 1995). Based on partial peptide sequences of the rice LEA protein, the rice LEA protein showed an overall similarity of 72% to the group 3 LEA proteins from barley (Hong et al., 1988) and wheat (Curry et al., 1991). The rice, wheat, and barley group 3 LEA proteins have similar electrophoretic mobility.

In this study, we have taken a transgenic approach to investigating the function of the HVA1 protein in stress protection. Constitutive and high-level expression of the barley *HVA1* gene in transgenic rice plants allowed us to test the role of the HVA1 protein in stress tolerance. We found that accumulation of the barley HVA1 protein in the vegetative tissues of transgenic rice plants conferred increased tolerance to water deficit and salt stress and that the extent of stress tolerance correlated with the level of HVA1 protein accumulation. Our results not only demonstrate a role for the barley LEA protein in stress protection but also suggest the potential usefulness of LEA genes for genetic engineering of stress tolerance.

MATERIALS AND METHODS

Construction of the *Act1-HVA1* Plasmid for Rice Transformation

A 1.0-kb *Eco*RI fragment containing the full-length *HVA1* cDNA was isolated from the cDNA clone *pHVA1* (Hong et al., 1988), and this fragment was blunted with Klenow DNA polymerase and subcloned into the *Sma*I site of the plasmid expression vector pBY505 (Wang and Wu, 1995), which is a derivative of pBluescriptIIKS(+) (Stratagene), to create pBY520. On pBY520, the *HVA1* fragment is located between the rice *Act1* promoter and the potato *Pin2* 3′ region. Plasmid pBY520 also contains the bacterial phosphinothricin acetyl transferase structural gene (commonly known as the *bar* gene), which serves as the selectable marker in rice transformation by conferring resistance to phosphinothricin-based herbicide. The *bar* gene is regulated by the CaMV 35S promoter and followed by the nopaline synthase gene termination signal.

Production of Transgenic Rice Plants

Calli were induced from immature embryos of rice (*Oryza sativa* L. cv Nipponbare), and suspension cultures were established from selected embryogenic calli after 3 months of subculture in liquid medium. Fine suspension culture cells were used as the transformation material and bombarded with tungsten particles coated with the pBY520 plasmid, essentially as described by Cao et al. (1992). Resistant calli were selected in selection medium, containing 6 mg L^{-1} ammonium glufosinate (Crescent Chemical Co., Hauppauge, NY) as the selective agent, for 5 to 7 weeks. The resistant calli were transferred to MS (Murashige and Skoog, 1962) regeneration medium containing 3 mg L^{-1} ammonium glufosinate to regenerate into plants. Plants regenerated from the same resistant callus were regarded as clones of the same line. Regenerated plants were transferred into soil and grown in the greenhouse (32°C day/22°C night, with a supplemental photoperiod of 10 h).

Herbicide-Resistance Test of Transgenic Rice Plants

The presence of the transferred genes in regenerated rice plants was first indicated by the herbicide resistance of the plants. For the herbicide-resistance test, a water solution containing 0.5% (v/v) commercial herbicide Basta (containing 162 g L^{-1} glufosinate ammonium; Hoechst-Roussel Agri-Vet Co., Somerville, NJ) and 0.1% (v/v) Tween-20 was painted on both sides of a leaf. After 1 week, the resistant/sensitive phenotype was scored.

DNA Blot Hybridization Analysis of Transgenic Rice Plants

Integration of the transferred genes into the rice genome was confirmed by DNA blot hybridization analysis using the *HVA1*-coding region as the probe. Genomic DNA was isolated as described by Zhao et al. (1989). For DNA blot hybridization analysis, 10 to 15 μg of DNA from each sample were digested by a suitable restriction endonuclease, separated on a 1.0% agarose gel, transferred onto a nylon membrane, and hybridized with the ^{32}P-labeled *HVA1* probe.

Immunoblot Analysis of HVA1 Protein Production in Transgenic Rice Plants

Protein extracts were prepared by grinding plant tissue in liquid nitrogen and homogenizing it in extraction buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% (v/v) Triton X-100, 0.1% (w/v) Sarkosyl, 10 mM β-mercaptoethanol, and 25 mg mL^{-1} PMSF. Mature seeds were cut into halves, and the embryo-containing

half-seeds were directly ground into a fine powder and homogenized in the same extraction buffer. The homogenates were centrifuged at 5,000*g* for 5 min at room temperature. The supernatants were further clarified by centrifugation at 12,000*g* for 15 min at 4°C. The protein concentrations were determined, based on the method of Bradford (1976), using a dye concentrate from Bio-Rad. Proteins were separated by SDS-PAGE minigels, transferred electrophoretically to a PVDF membrane using Mini Trans-Blot Cells (Bio-Rad), blocked with 3% (w/v) BSA in Tris-buffered saline containing 0.05% (v/v) Triton X-100, incubated with rabbit anti-HVA1 antibody, and then incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Bio-Rad). The secondary antibody was detected using 4-nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate supplied in a alkaline phosphatase immunoassay kit from Bio-Rad. Immunoreaction signals on the blot filters were scanned using a densitometer (Helena Laboratories, Beaumont, TX) to quantify the relative amounts of the HVA1 protein. Partially purified HVA1 protein was used as the standard to estimate the levels of HVA1 protein in transgenic rice tissues.

Analysis of Growth Performance of Transgenic Plants under Water-Deficit and Salt-Stress Conditions

Evaluation of the growth performance under water-deficit and salt-stress conditions was carried out using the R_1 plants from three transgenic lines. These R_1 plants represent a mixed population that includes homozygous and heterozygous transgenic plants and segregated nontransgenic plants. Seeds of NT plants were used as control materials in all stress experiments. They are both referred to as NT plants in the text.

Seed Germination and Seedling Growth in Medium

Thirty R_1 seeds from each of three transgenic rice lines and two NT plants were surface sterilized and germinated in the dark at 25°C on three kinds of agarose media: MS, MS plus 100 mM NaCl, and MS plus 200 mM mannitol. The MS medium contained only its mineral salts. After 5 d, seeds were transferred to the MS medium. To test the response of young seedlings to stress conditions, seeds were germinated in the MS medium for 5 d. The 5-d-old seedlings were then divided, transferred onto two layers of Whatman paper in deep Petri dishes, and supplied with liquid MS, or MS plus 100 mM NaCl, or MS plus 200 mM mannitol. Seedlings were grown under light at 25°C, and the response to the stress conditions was monitored for 5 d.

Growth and Stress Treatments of Plants in Soil

Refined and sterilized field soil, supplemented with a composite fertilizer, was used to grow rice plants in the greenhouse. Seeds were germinated in MS medium for 7 d, and the 7-d-old seedlings were transferred into soil in small pots with holes in the bottom (8 × 8 cm, one plant per pot). The pots were kept in flat-bottom trays containing water. The seedlings were grown for an additional 2 weeks before they were exposed to stress treatments. At this stage, most of the 3-week-old seedlings had three leaves, and some seedlings had an emerging fourth leaf. Two stress experiments, using a different set of R_1 plants from the same R_0 transgenic lines, were conducted. In each experiment, 10 transgenic plants and at least 10 NT plants were used for each treatment. (a) Nonstress: The plants were supplied with water continuously from the trays. (b) Water stress: To start water deficit, water was withheld from the trays for 5 d. The absolute water content of the soil decreased from 28% (saturated level) to 10% during the 5-d period. The stressed plants were then resupplied with water for 2 d to allow the wilted plants to recover. Then, a second round of water stress was carried out. The water content of the soil was determined by measuring the wet weight and dry weight of soil samples taken from additional pots containing NT plants under the same stress conditions. (c) Salt stress: Short-term, severe salt stress in the soil was produced by transferring the pots into trays containing 200 mM NaCl solution for 10 d. Then, the pots were transferred back to trays containing tap water to let the plants recover for 10 d. The salt concentration in the soil was quickly reduced by flushing the soil in the pots from the top with water and changing the water in the trays several times during the first 2 d. After 10 d of recovery, a second round of salt stress was imposed by transferring the pots into trays filled with 50 mM NaCl solution for 30 d.

Data Collection and Statistical Analysis of Growth Performance

Before stress treatments were started, the initial plant height, leaf number, and leaf length of each NT plant and transgenic plant were measured. During and after stress treatments, each plant was again evaluated. For statistical analysis, the mean value of the 10 tested plants in each treatment was calculated and used for comparing the transgenic plants with the NT plants. Although the R_1 plant population probably included segregated nontransgenic plants, they were all treated as transgenic plants in data collection and statistical analysis.

RESULTS

Production and Molecular Analysis of Transgenic Rice Plants

The structure of the plasmid pBY520 is shown in Figure 1. The cDNA of the barley LEA gene, *HVA1*, is located downstream of the rice *Act1* promoter. The coding region of the *bar* gene is located downstream of the CaMV 35S promoter. Rice suspension cells, supported by filter papers and precultured in solid medium, were bombarded by tungsten particles coated with the plasmid DNA pBY520. Results of three transformation experiments are summarized in Table I. Thirty-three plates of suspension cells were bombarded in these transformation experiments. Two hundred ammonium glufosinate-resistant calli were selected and transferred onto regeneration medium. Sixty-three independent lines of plants (120 plants) were regenerated and grown in the greenhouse. As shown in Table I, 86% of the transgenic plants were fertile, producing various num-

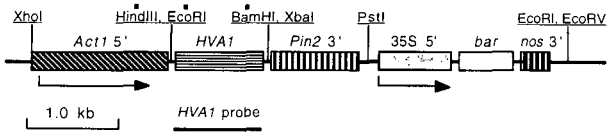

Figure 1. Structure of the plasmid pBY520 for expression of *HVA1* in transgenic rice. Expression of the *HVA1* structural gene is regulated by the rice *Act1* promoter and the potato *Pin2* 3′ region. The bacterial phosphinothricin acetyl transferase structural gene (*bar*), which is regulated by the CaMV 35S promoter and the nopaline synthase gene (*nos*) 3′ region, serves as the selectable marker in rice transformation. The two gene expression cassettes are cloned on the plasmid pBluescriptIIKS(+), as described in "Materials and Methods." Only common restriction endonucleases sites are indicated, and those sites used for DNA digestion in DNA blot hybridization are marked with a filled square. The DNA fragment used as a probe in DNA blot hybridization is also indicated.

bers of seeds. The sterility of some transgenic lines appeared unrelated to the presence of the foreign genes, since similar percentages of sterile plants were obtained in parallel experiments in which the suspension cells were bombarded without plasmid DNA or with several other gene constructs.

Phosphinothricin acetyl transferase encoded by the *bar* gene can detoxify phosphinothricin-based herbicides. Twenty-nine lines of plants were first tested for herbicide resistance. When painted with 0.5% commercial herbicide Basta, the leaves of transgenic plants showed complete resistance, whereas the leaves of nontransformed plants turned yellow and died. Among 29 lines of plants that were tested for herbicide resistance, 90% of them were resistant. The same 29 lines were further analyzed by DNA blot hybridization using the *HVA1* cDNA fragment as the probe. Data of DNA blot hybridization of 15 transgenic plants are shown in Figure 2. Digestion of plasmid pBY520 or genomic DNA from transgenic rice plants releases the 1.0-kb fragment containing the *HVA1* coding region. Among 29 lines analyzed, 23 of them contained the expected 1.0-kb hybridization band, although most transgenic plants also contained extra hybridization bands of varying sizes that may represent rearranged copies of the *HVA1* fragment. The hybridization patterns of all transgenic plants were unique, except the predicted 1.0-kb hybridization band. This suggests that these transgenic lines were derived from independent transformation events. Results of DNA blot hybridization were generally consistent with those of the herbicide-resistance test, which suggests that both the selectable marker gene and the *HVA1* gene on the same plasmid were efficiently co-integrated into the rice genome.

Analysis of Accumulation of HVA1 Protein in R_0 Transgenic Rice Plants

We analyzed the accumulation of HVA1 protein in a number of transgenic lines that were selected based on the DNA blot hybridization data. Protein extracts were prepared from both leaf and root tissues. The HVA1 protein was detected by a polyclonal antibody raised against purified barley HVA1 protein. A single band of 27 kD in SDS-PAGE gel, which corresponds to the HVA1 protein, was detected in the leaf tissue of different transgenic lines, as shown in Figure 3A. Accumulation of HVA1 protein was also readily detected in roots as shown in Figure 3B, although the levels were lower than those in the leaf tissues. The relative levels of accumulation of the HVA1 protein in roots correspond to those in leaf tissue among different transgenic lines. Protein extracts of nontransformed plants did not show the 27-kD protein band, and there were no additional bands of other sizes detected in the protein extracts of the transgenic plants or the nontransformed plants. Using a partially purified HVA1 protein preparation as the standard, we estimated the levels of HVA1 protein accumulated in the leaf and root tissues of different transgenic lines to be in the range of 0.3 to 2.5% of the total soluble proteins (Table II).

The 27-kD band corresponding to the HVA1 protein was not detected in the protein extracts of mature transgenic seeds. However, two strong bands with lower molecular mass, 20 and 13 kD respectively, were detected in both the control and transformed plants, as shown in Figure 3C. Since an mRNA transcript highly homologous to the barley *HVA1* gene has already been detected in mature rice seeds in a previous study (Hong et al., 1992), these two proteins may represent endogenous rice LEA or LEA-like proteins that accumulate during the late stage of seed development.

Increased Tolerance to Water Deficit and Salt Stress of Transgenic Rice Plants

The results described above demonstrated that expression of the barley *HVA1* regulated by the strong rice *Act1* promoter led to high-level accumulation of the HVA1 protein in vegetative tissues of transgenic rice plants. As described earlier, 86% of the primary transgenic plants were fertile. These transgenic rice plants also appeared morphologically normal compared with transformation procedure-derived, NT plants. Taken together, these results suggest that accumulation of HVA1 protein does not have detrimental effects on the growth and development of rice plants.

Table I. *Summary of transformation experiments*

Transformation Experiment No.	No. of Plates of Cells Bombarded	No. of Resistant Calli Selected	No. of Lines (Plants) Regenerated	No. of Fertile Lines (%)
1	8	107	27 (67)	
2	15	69	15 (27)	
3	10	24	21 (26)	
Total	33	200	63 (120)	54 (86)

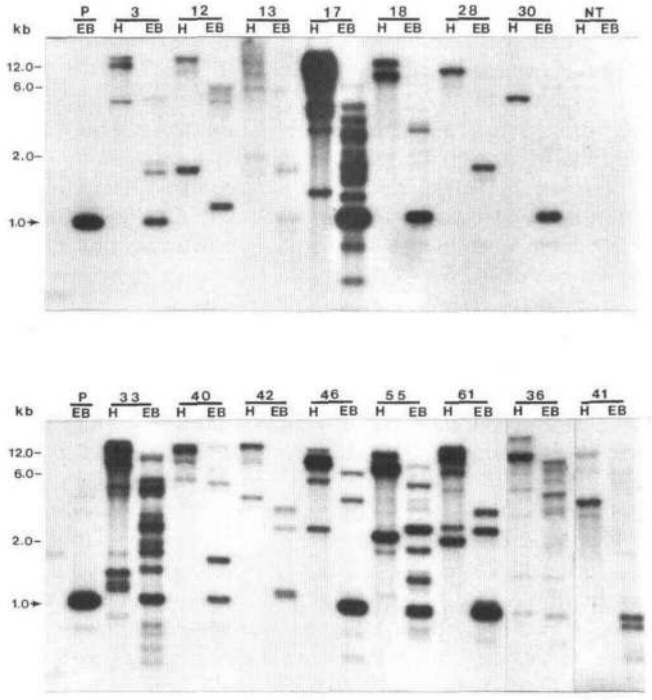

Figure 2. DNA blot hybridization analysis to show integration of the *HVA1* gene into the genome of the R_0 transgenic rice plants. Ten micrograms of genomic DNA from each plant were digested with *Hin*dIII (H) or with a combination of *Eco*RI and *Bam*HI (EB), separated on a 1% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled *HVA1* cDNA fragment, as shown in Figure 1. There is a single *Hin*dIII site on the plasmid; thus, digestion of genomic DNA with *Hin*dIII releases the fusion fragment containing the *HVA1* sequence and the rice genomic sequence. Digestion with *Eco*RI and *Bam*HI releases the 1.0-kb fragment containing the *HVA1* cDNA. The identification numbers of transgenic lines are marked on the top. The DNA molecular size markers are shown in kb on the left. The 1.0-kb hybridization band, corresponding to the *HVA1* cDNA fragment, is marked with an arrow. P, Plasmid pBY520 digested with *Eco*RI and *Bam*HI.

We were interested in learning whether the high-level accumulation of the HVA1 protein would have any beneficial effect on the growth performance of transgenic rice plants under water- and salt-stress conditions. Evaluation of growth performance was carried out using the R_1 plants. Seeds of wild-type rice plants or seeds of transformation procedure-derived, NT plants were used as controls. No significant difference was observed between wild-type controls and transformation procedure-derived NT plants in their growth performance under nonstressed or stressed conditions. Thus, transformation procedure-derived, NT plants served as good controls for the stress experiments (data of wild-type controls were not included in this report).

Seed Germination and Seedling Growth in Medium under Osmotic and Salt-Stress Conditions

In the MS medium, at least 90% of both transgenic and control seeds germinated well, and no difference was observed in their seedling growth. In MS plus 100 mM NaCl or MS plus 200 mM mannitol, no difference was observed between transgenic and control seeds, both of which germinated slowly (2-d delay for emergence of the shoot and root). After 5 d in the stress medium, the germinated seeds (with 0.2- to 0.5-cm-long shoot) were transferred onto MS medium. Both transgenic and control seedlings recovered and resumed normal growth, but transgenic seedlings grew faster during this recovery period, their shoots were significantly longer after 1 week (Table III), and they had one to three more adventitious roots than the control seedlings (data not shown). No significant difference was observed between NT plants and transgenic plants when seeds were germinated and grown continuously in the MS medium (Table III).

Five-day-old seedlings from seeds germinated in the MS medium were tested for their response to salt stress. Both the transgenic and control seedlings were very sensitive to

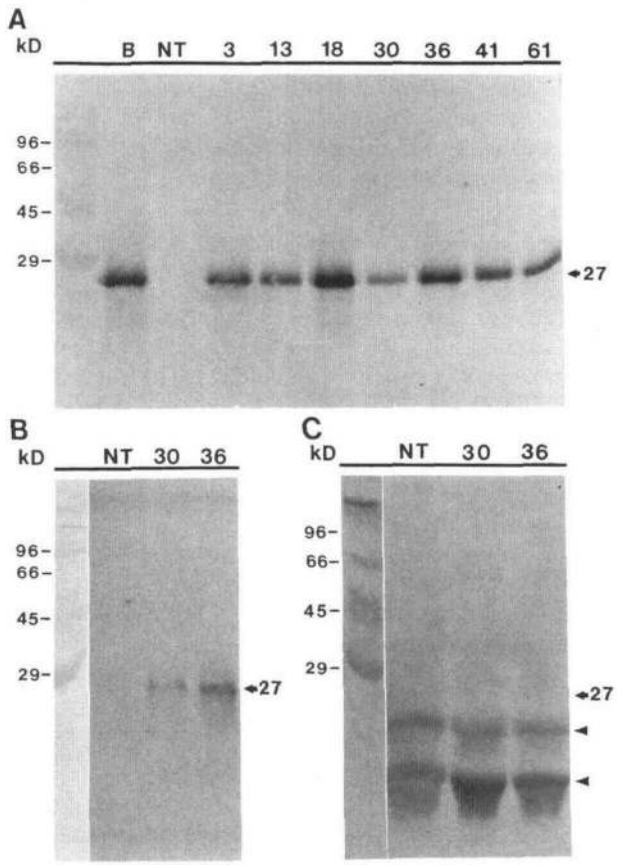

Figure 3. Immunoblot detection of HVA1 protein accumulation in the R_0 transgenic rice plants. Equal amounts of total soluble proteins (20 μg) were separated on 12% SDS-PAGE and immunoblot detection was performed as described in "Materials and Methods." The identification numbers of transgenic lines are indicated on the top. Molecular mass markers are indicated in kD on the left. The HVA1 protein shown as a 27-kD band is indicated by an arrow at the right. A, HVA1 protein accumulation in leaves. Lane B contains purified HVA1 protein as the positive control. B, HVA1 protein accumulation in roots. C, HVA1-like protein accumulation in mature rice seeds. The two rice proteins cross-reacting with the HVA1 antibody are marked with arrowheads on the right.

Table II. *Estimated levels of HVA1 protein accumulation in different transgenic lines*

ND, Not determined.

Transgenic Line (R_0)	Level of HVA1 Protein Accumulation	
	Leaf	Root
	% of total soluble proteins	
NT	0	0
3	1.00	ND
13	0.75	ND
18	2.50	ND
19	0.60	0.30
30	0.50	0.30
36	1.50	1.00
38	0.80	0.60
41	1.00	0.70
61	0.75	ND

salt stress. In MS plus 100 mM NaCl, the seedlings gradually wilted within 1 week. However, the wilting of transgenic seedlings was delayed compared to the control seedlings. During the first 3 d in MS plus 100 mM NaCl, 18 of 30 control seedlings became wilted, but only 7 of 60 R_1 transgenic seedlings from three transgenic lines became wilted (data not shown).

Growth Performance of Transgenic Plants in Soil under Water-Deficit Conditions

The above experiments showed that transgenic seedlings and control seedlings respond to stress treatments differently. Extensive stress experiments were conducted using 3-week-old plants grown in the soil. Under constant nonstress conditions in soil, no significant differences were observed between transgenic plants and control plants in their growth performance during the entire period of the experiment.

Upon withholding water from the trays, the rapid decrease of water content in the soil created a water-deficit condition. The absolute water content of the soil decreased from 28% (saturated level) to 10% after 5 d of withholding water. There was a significant difference between the transgenic plants and the control plants in their response to this water-deficit condition. Leaves at the same developmental stage of transgenic plants wilted about 1 to 2 d later than those of control plants. After 4 to 5 d of water stress, leaves of both control and transgenic plants were wilted, but wilting of transgenic plant leaves was less severe. The difference between transgenic and control plants in response to water deficit was also reflected in the growth rate of young leaves (increase of leaf length) during the first 3 d of water stress. Water deficit inhibited the growth of the young leaves of both control and transgenic plants. However, transgenic plants maintained higher growth rates (shown as percentage of leaf length increase) than did control plants (Table IV). After the water-stressed plants were rewatered, the transgenic plants showed better recovery and resumed faster growth than the control plants (data not shown). As shown in Figure 4A, transgenic plants of lines 36 and 41 were less damaged by the water stress and looked much healthier, whereas older leaves and tips of young leaves of NT plants showed poor recovery and gradually died. Data in Table IV show the average plant height and root fresh weight of the stressed plants after four cycles of a 5-d water deficit followed by a 2-d recovery with watering. Both plant height and root fresh weight were significantly higher in transgenic plants than in control plants. In summary, transgenic plants showed significant advantages over control plants in their growth performance under water-stress conditions, particularly in the growth of roots.

Table III. *Seed germination and growth of young seedlings in medium under osmotic stress or salt stress*

Data were collected 12 d after seed germination: 5 d in stress medium (MS plus 200 mM mannitol or MS plus 100 mM NaCl) and 7 d in nonstress medium (MS). Each value ± SE represents the average of 10 seedlings. For nonstress control, seeds were germinated and grown continuously in the MS medium for 12 d. Numbers in parentheses are the percentages of shoot length of transgenic seedlings compared to control seedlings, which was taken as 100.

Transgenic Line	Length of Shoot		
	MS	MS + mannitol	MS + NaCl
		cm	
NT	7.5 ± 0.2	4.2 ± 0.2 (100)	2.7 ± 0.2 (100)
30	7.3 ± 0.2	5.2 ± 0.2 (124)	3.5 ± 0.2 (130)
36	7.4 ± 0.2	6.1 ± 0.2 (145)	4.9 ± 0.2 (181)
41	7.7 ± 0.2	5.9 ± 0.2 (140)	4.0 ± 0.2 (148)

Growth Performance of Transgenic Plants in Soil under Salt-Stress Conditions

Severe salt stress (200 mM NaCl) significantly inhibited the growth of both transgenic and control plants. However, transgenic plants maintained a much higher growth rate than the control plants at the early stage (d 0 to d 5) of salt

Table IV. *Growth performance of transgenic rice plants in soil under water-stress conditions*

The mean length of the two longest leaves at the top of the plants was used as a measure of the plant height. Each value ± SE represents the average of 10 plants except for root fresh weight, which is the average of four plants. Numbers in parentheses are the percentages of transgenic plants compared to control plants, which was taken as 100.

Transgenic Line	Leaf Growth Rate[a]	Plant Height[b]	Root Fresh Wt[b]
	% length increase[a]	*cm*	*g*
NT	69	22 ± 1.4 (100)	0.9 ± 0.1 (100)
30	90	29 ± 1.1 (132)	1.4 ± 0.1 (156)
36	129	37 ± 1.8 (168)	2.1 ± 0.1 (233)
41	113	33 ± 1.8 (150)	2.3 ± 0.3 (256)

[a] The lengths of the two upper leaves were measured before and 3 d after withholding water from the trays. The growth rate was calculated as the percentage length increase of the two leaves during the 3-d period of water stress. [b]Data were collected 28 d after the beginning of initial water stress (four cycles of 5 d of water stress followed by 2 d of recovery with watering).

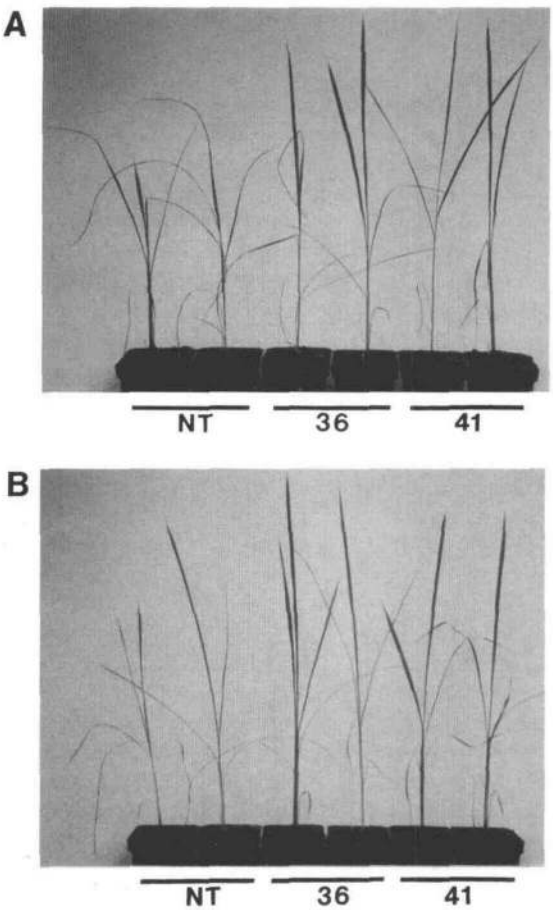

Figure 4. Comparison of transgenic plants and NT plants grown under drought and salinity stress conditions. Two NT plants and two R_1 transgenic plants from each of two lines (numbers 36 and 41) are show here. A, Plants recovered from drought stress. Photograph was taken 21 d after the beginning of initial water stress (three cycles of 5 d of drought stress followed by 2 d of recovery with watering). B, Plants recovered from salt stress. Photograph was taken after 10 d of salt stress in 200 mM NaCl and 10 d of recovery in tap water.

stress (Table V). Early symptoms of damage due to salt stress, such as wilting, bleaching, and death of leaf tips, occurred first in older leaves. Leaves at the bottom of a plant wilted or died first. At the later stage, the young leaves developed necrosis symptoms and started to wilt and dry from the leaf tips. Appearance and development of these symptoms occurred much more slowly in transgenic plants than in control plants. At the time when the two bottom leaves of most control plants wilted, the lowest leaf of most transgenic plants showed only slight wilting. Wilting of young leaves of transgenic plants was always less severe compared with control plants. Upon removal of the salt stress, transgenic plants of lines 36 and 41 showed much better recovery than the NT plants (Fig. 4B). Data in Table V show the average shoot height and root fresh weight of the stressed plants 30 d after the initial salt-stress treatment. Again, transgenic plants showed significantly better performance than did the control plants under extended stress condition. Under continuous salt stress, all 10 NT plants gradually died, whereas 6 to 8 of 10 R_2 transgenic plants survived (Table V).

Analysis of Accumulation of HVA1 Protein in R_1 Transgenic Rice Plants

At the end of the stress experiment, HVA1 protein accumulation was analyzed in R_1 plants from two R_0 transgenic lines: numbers 36 and 41. Eight R_1 plants from each of the two R_0 transgenic lines were analyzed. In each line, HVA1 protein was not detected in two of eight analyzed R_1 plants, as shown in Figure 5, because of the segregation of the transferred gene in these R_1 plants. Those R_1 plants that lacked HVA1 protein accumulation were severely inhibited and damaged by the stress treatments, showed poor recovery after the first period of salt stress, and gradually died under continuous stress condition. HVA1 protein accumulation was detected in all of the surviving R_1 transgenic plants that showed tolerance to stress.

DISCUSSION

We found that high-level accumulation of the HVA1 protein in the transgenic rice plants confers significantly

Table V. *Growth performance of transgenic rice plants in soil under salt-stress conditions*

Transgenic Line	Leaf Growth Rate[a]	Plant Height[b]	Root Fresh Wt[b]	No. of Surviving Plants[c]
	% length increase[a]	*cm*	*g*	
NT	76	19 ± 1.1 (100)	1.2 ± 0.1 (100)	0
30	90	23 ± 0.9 (121)	1.9 ± 0.1 (158)	6
36	103	29 ± 0.8 (153)	ND	8
41	115	26 ± 0.8 (137)	2.6 ± 0.1 (217)	8

[a] The lengths of the two upper leaves were measured before salt stress and at 5 d after salt-stress condition was imposed. The growth rate was calculated as the percentage length increase of the two leaves during the 5-d period of salt stress. [b] Data were collected 30 d after the beginning of the initial salt stress (10 d in 200 mM NaCl, 10 d in tap water for recovery, and 10 d in 50 mM NaCl). The mean length of the two longest leaves on the top of the plants was used as a measure of the plant height. Each value ± SE represents the average of 10 plants except for root fresh weight, which is the average of four plants. Numbers in parentheses are the percentages of transgenic plants compared to control plants, which was taken as 100. ND, Not determined. [c] Data were collected from a second stress experiment 40 d after beginning of the initial salt stress (10 d in 200 mM NaCl, 10 d in tap water for recovery, and 20 d in 50 mM NaCl). Ten transgenic plants from each transgenic line and 10 NT plants were used.

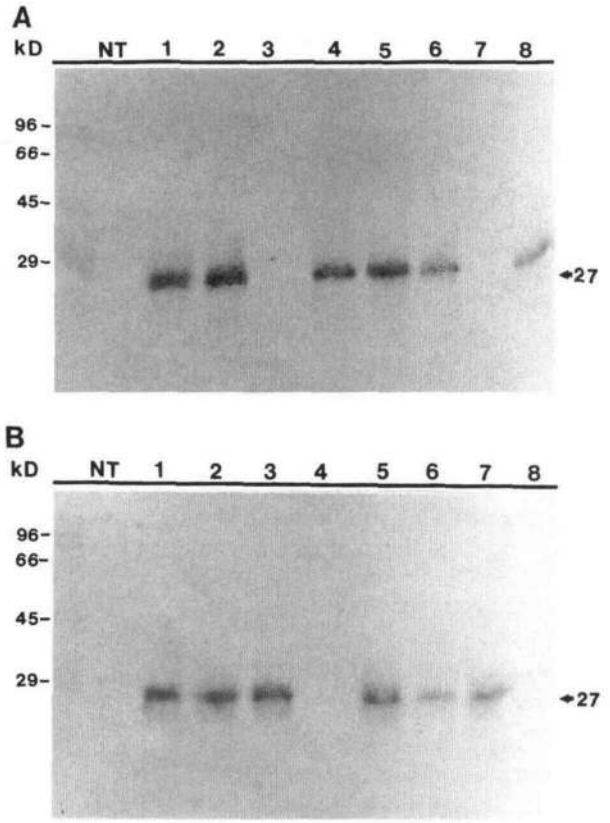

Figure 5. Immunoblot detection of HVA1 protein accumulation in leaves of the R_1 transgenic plants from two primary (R_0) transgenic lines. The experimental conditions are the same as for Figure 3. The identification numbers of R_1 plants are indicated on the top. Molecular mass markers are indicated in kD on the left. The HVA1 protein shown as a 27-kD band is indicated by an arrow at the right. A, R_1 plants from transgenic line number 36. B, R_1 plants from transgenic line number 41.

increased tolerance to water deficit and salt stress. Transgenic plants maintained higher growth rates than NT plants under both water-deficit and salt-stress conditions. Appearance and development of the major damage symptoms (wilting, dying of old leaves, and necrosis of young leaves) caused by the two stress conditions were delayed in transgenic plants. When the stress conditions were removed, the transgenic plants showed better recovery than did the control plants. Plants from all three tested transgenic lines showed much better performance than NT plants under stress conditions. The increased stress tolerance in R_1 plants correlated with the expression of the HVA1 protein. In comparing R_1 plants from different transgenic lines, we found plants from lines 36 and 41 showed better performance than plants from line number 30. The better performance of plants from lines 36 and 41 seems to be correlated with higher levels of HVA1 protein accumulation in their R_0 plants (Fig. 3A; Table II). These results strongly support the hypothesized role of LEA protein in water-stress protection. Such an in vivo protective role also suggests the potential usefulness of LEA protein genes as molecular tools for genetic engineering of stress tolerance.

Data from a number of previous studies indicate that accumulation of group 3 LEA proteins are correlated with stress tolerance (Ried and Walker-Simmons, 1993; Moons et al., 1995). On the other hand, the presence of other LEA proteins is not always correlated with stress tolerance. For example, comparative studies of wild rice and paddy rice showed that the intolerance of wild rice seeds to dehydration at low temperature is not due to an absence of, or an inability to synthesize, group 2 LEA/dehydrin proteins, ABA, or soluble carbohydrates (Bradford and Chandler, 1992; Still et al., 1994). Overproduction of a group 2 LEA protein from the resurrection plant *Craterostigma* in tobacco did not confer tolerance to osmotic stress (Iturriaga et al., 1992). These studies suggest that certain specific LEA proteins may not be responsible for desiccation tolerance or their presence alone is not sufficient to prevent injury under desiccation conditions. It has been found that LEA proteins are not sufficient to confer desiccation tolerance in soybean seeds but that LEA proteins together with soluble sugars contribute to the tolerance (Blackman et al., 1991, 1992).

A group 2 LEA protein and its cDNA have already been characterized from rice (Mundy and Chua, 1988). The four members of a group 2 LEA gene family are tandemly arranged in a single locus and are coordinately expressed in various rice tissues in response to ABA, desiccation, and salt stress (Yamaguchi-Shinozaki et al., 1989). However, the functions of these LEA proteins are unknown. Recently, both group 2 and group 3 LEA proteins have been characterized from salt-tolerant Indica rice varieties, and the accumulation of these LEA proteins in response to ABA and salt stress was correlated with varietal tolerance to salt stress (Moons et al., 1995). Recent studies showed that group 2 LEA genes are present in many plant species, but the expression of these genes is differentially regulated in stress-sensitive and -tolerant species (Close et al., 1993; Danyluk et al., 1994). As more LEA genes are characterized and their functions in stress tolerance are confirmed, manipulation of endogenous LEA gene expression, in combination with introduction of additional stress tolerance genes, may prove to be an efficient strategy for genetic crop improvement.

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