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HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (*Oryza sativa* L.) via cell membrane protection

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Abstract

Drought is by far the leading environmental stress-limiting crop yields world-wide. Genetic engineering techniques hold great promise for developing crop cultivars with drought tolerance. Transgenic rice plants have been developed by engineering a wide variety of genes and were shown to be drought tolerant. Understanding the mechanism of stress tolerance in these transgenic plants under agronomically realistic stress conditions would further hasten breeding for drought resistance in rice. In this study, transgenic rice lines expressing the barley *HVA1* gene were tested under prolonged drought stress cycle to understand the mechanism of dehydration tolerance. Transgenic plants maintained higher leaf relative water content (RWC) and showed lesser reduction in plant growth under drought stress as compared to non-transgenic (NT) plants. Maintenance of higher plant water status delayed wilting by more than 2 weeks in transgenic plants as compared to NT plants. Transgenic lines had relatively better cell membrane protection than NT line 28 days after stress. Both transgenic and NT lines had similar but low levels of osmotic adjustment (OA) under drought stress. The results indicated that the production of HVA1 proteins might have helped in better performance of transgenic rice plants by protecting cell membrane from injury under drought stress.
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Keywords: Transgenic rice; Dehydration tolerance; LEA protein; *HVA1* gene; Cell membrane protection; Osmotic adjustment

1. Introduction

Rainfed rice is grown on 69 million hectares, representing more than 45% of the total area planted to rice [1]. These areas frequently experience severe water deficit due to uncertain and uneven rainfall distribution patterns. Drought is by far the leading environmental stress-limiting rice productivity in rainfed ecosystems. Developing rice cultivars with an inherent capacity to withstand drought stress would help to stabilize rainfed rice production. However, progress in genetic improvement of rice for water-limiting environments has been slow and more limited [2], due to lack of knowledge about the mechanism of tolerance, poor understanding of the inheritance of tolerance, low heritability and a lack of efficient techniques for screening breeding materials for drought tolerance [3]. Understanding the genetic and physiological basis of drought tolerance is fundamental to enable breeders and molecular biologists to develop rice cultivars suitable for water-limiting environments. Though conventional breeding has had a limited success in improving yields of crops grown in stressful environments, there is a growing belief that further gains can only be achieved through targeted manipulation of genes involved in stress resistance [4]. Genetic engineering has undoubtedly opened a new avenue to overcome crop losses due to various abiotic stresses prevalent in the agricultural ecosystems [5–7]. Considerable progress has been made in developing transgenic rice lines tolerant to drought stress [8–15]. Results indicate the potential usefulness of *LEA* genes as molecular tools for genetic improvement of rice for water-limiting environments [8,9,16]. Transgenic rice plants containing *HVA1* gene from barley were shown to be tolerant to short but rapid water

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stress cycles [8]. The levels of HVA1 protein under water stress correlated with stress tolerance of the transgenic plants. Further progress in making these transgenic plants available to practical agriculture, however, depends on understanding the stress protection mechanism in the transgenic plants. Despite extensive studies, our knowledge of the biochemical function of LEA proteins is still rudimentary [17]. The exact mechanism of stress tolerance due to the expression of *LEA* genes need to be studied for targeted engineering and pyramiding of different stress protection pathways to substantially increase plant drought tolerance [18].

The use of transgenics to provide enhanced drought tolerance is still experimental in nature, though progress is being made [19]. The obvious next step is to investigate the impact of the introduced gene by measuring the growth and yield of transgenic plants in field environment. However, there is a need to investigate thoroughly the physiology of the transgenic plants under agronomically realistic stress conditions before such approaches can be applied in the field [4]. The desiccation stress applied by most researchers while evaluating transgenic plants for drought tolerance is 'shock' treatments. The plants were grown in small pots with unrealistically less soil volume and were subjected to rapid stress cycles each ranging from an hour to several days [11–15]. For instance, drought tolerance of transgenic rice was evaluated by drying seedlings in air for 1 h [15]. When stress is imposed rapidly a greater number of responses will be injury-induced than under a slower long-term application of water-deficit stress [20]. Three important elements of drought characterization for successful breeding of stress tolerance are timing, duration and intensity of stress [21]. For most crops, including rainfed rice, drought tends to develop slowly as the soil dries. Plants that are subjected to drought conditions in this gradual manner accumulate solutes that maintain cell hydration, and undergo complex adjustments in their morphology and physiological characteristics. Since most experiments that have been published thus far are based on a rapid, severe water-deficit treatment, it is important that experiments be conducted under conditions that more closely approximate stress development in the field [22]. Such an experiment will permit a better understanding of the potential functions of the introduced gene in stress tolerance.

Transgenic plants have the potential to be powerful and to aid in helping us understand and manipulate the responses of plants to stress, but they can only be so when studied with the help of a sound background in stress physiology [23]. Uncertainties between causes and effects persist in the study of drought tolerance using transgenic materials making conclusions at times erroneous and incorrect [23,24]. A recent example is the study of transgenic tomato showing enhanced resistance to water-deficit stress [25]. The results indicate that CBF1 reduces water use in potted plants because it conferred a small phenotype and not due to stress tolerance in the transgenic plants. The effect of CBF1 on physiological or biochemical factors ascribing drought tolerance must be explored in isolation of its effect on plant morphology [26]. Secondly, "% water content" in leaves (on a dry matter basis) is used as the measure of plant water status [25]. This measure cannot be used to assess plant water status because differences in leaf assimilation and structure between genotypes can affect % water content. The standard tests for plant water status are 'relative water content (RWC)' or 'leaf water potential (LWP)'. Thus, the contribution of these studies is considerably weakened by the lack of understanding of the mechanism of stress protection and poor appreciation of the water-relations measurements that are necessary to document and interpret whole-plant responses to low water-potential environment [24]. Thus, the objective of the present study was to understand the principal mechanism of HVA1, a group three LEA protein in conferring drought tolerance in transgenic rice plants under prolonged drought stress cycle.

2. Materials and methods

2.1. Plant material

The rice cultivar, Nipponbare was used for transformation with the barley *HVA1* gene. Construction of the *Act1-HVA1* plasmid for production of transgenic plants, DNA-blot hybridization analysis of transgenic rice plants and immunoblot analysis of HVA1 protein production in transgenic rice plants were earlier described [8]. Expression of the transgene was under the constitutive control of rice *Act1* promoter. From the several transgenic rice lines produced, line #30–54 and 30–59 were chosen for the present study since these two lines consistently produced moderate levels of HVA1 protein (0.5% of total soluble proteins in the leaf) both in R_1 and R_2 generations [27]. R_2 generation plants of these two transgenic lines were used in the present experiment.

2.2. Plant growth and drought stress conditions

The experiment was conducted in a temperature-controlled greenhouse at Texas Tech University, Lubbock, TX, USA. The two transgenic (#30–54 and 30–59) and the non-transgenic (NT) lines were raised in large volume (18 l capacity) polyvinyl pots (30 cm diameter at the top, 26 cm diameter at the bottom and 31 cm height) each filled with 6 kg of air dried commercial potting mixture containing peat moss, perlite, bark and vermiculite (Ball Growing-on mix-1, Ivy Gardens, Lubbock, TX, USA). This growing condition was earlier found to be suitable for a slow stress development cycle [28]. Two plants were established from seeds in each pot. Plants were watered daily up to 40 days after sowing (DAS) and supplemented with nutrient solution ('Miracle-Gro' liquid fertilizer, Port Washington, New York) in each 15 days interval. Drought stress was imposed by withholding water to the treatment pots. The last irrigation was applied to field capacity on the evening of 40 DAS

for the stress treatment plants. A set of plants in both transgenic and NT lines was maintained under non-stress condition with regular irrigation. Development of stress in the treatment plants was routinely monitored by visual symptoms of leaf rolling and leaf drying and by measurements on plant water status indicators, viz. leaf relative water content and leaf water potential at pre-determined intervals.

2.3. Plant water status, cell membrane injury and osmotic adjustment determinations

Measurements of RWC, LWP, leaf osmotic potential (OP) and cell membrane injury were made the day after last irrigation (non-stress measurements) and following this at 7 days interval until wilting of plants under stress treatment. Wilting was judged as complete rolling of the youngest leaves and desiccation of older leaves [29]. On each sampling date, leaves were sampled in the morning between 10 and 11 h and RWC, LWP, OP and cell membrane injury were determined using the midsection of the second-youngest fully expanded leaf blade. RWC was determined by the standard method [30], using 6 cm long midsection of leaf following a 4 h rehydration period. For OP, a sample consisting of three 1 cm long midleaf segments was sealed in a thermocouple psychrometer cup (2 ml volume) and freeze-killed at −80 °C. Prior to the measurement, samples were thawed for 30 min at room temperature. LWP was measured in similar leaf samples without freeze killing. Both OP and LWP were measured in a commercial Peltier type thermocouple psychrometer (Model SC-10A, Decagon Devices Inc., Pullman, Washington) after 2 h equilibration period with a cooling current for 15 s [31]. Five replications per rice line per sampling date were made for all the four variables. Osmotic adjustment (OA) was calculated as the difference in measured leaf OP between well-watered (non-stress) and water-stressed plants that are rehydrated [28,32]. For OA, both transgenic and NT plants were subjected to severe stress, RWC declining to less than 60%. Once this critical RWC is reached, leaves were sampled for RWC and LWP and the sampled plants were irrigated and leaf samples were collected next morning from rehydrated plants for measurement of OP. Cell membrane injury was determined following the standard protocol [33]. Briefly, three 3 cm long midleaf sections were cut and placed in a glass vial (20 ml) and washed three times in deionized water to remove electrolytes adhered on the leaf surface. Ten milliliter deionized water was added to the vial, capped and incubated in the dark for 24 h at room temperature. The conductance was measured using a conductivity meter (YSI Model 345, Yellow Springs, Ohio). After the initial measurement, the vials were autoclaved for 15 min to kill the leaf tissue and release all the electrolytes. After cooling, the final conductivity reading was taken. These two measurements were carried out individually for all the samples from both the control (non-stress) and stress treatments at 7 days intervals up to 28 days after stress. The control gave a measure of leakage solely due to the cutting and incubation of leaf discs. The conductance of the stress sample was a measure of electrolyte leakage due to water stress and was assumed to be proportional to the degree of injury to the cell membranes. Cell membrane injury was calculated following the formula, cell membrane injury $\% = [(T_1/T_2)/(C_1/C_2)] \times 100$, where T and C refer to the stress and control samples, respectively; the 1 and 2 refer to the initial and final conductance readings, respectively.

2.4. Plant dry weight determinations

Plants were sampled in non-stress and stress treatments at the end of the experiment (45 days after stress) from both transgenic and NT lines for dry weight determination. Six replicate pots per line per treatment were sampled for dry weight determinations. The two plants in a pot were pulled out gently along with roots, soils adhering to the roots were removed, root and shoot were separated at transition zone and dry weights determined after oven drying the tissues at 100 °C for 72 h.

3. Results and discussion

Maintenance of high (favourable) plant water status, as expressed in high RWC is an indication of drought resistance. However, variations among genetic materials in RWC could also be derived from differences in plant (shoot) size [34,35]. Constitutive expression of proteins may hamper the normal growth of transgenic plants resulting in smaller phenotypes as compared to NT plants. Small plants use less water than larger plants simply as a function of the respective difference in their leaf area. When grown in pots of a given volume, such as in this study and when irrigation is stopped to initiate water stress, larger NT plants will express wilting symptoms and stomatal closure earlier than the smaller transgenic plants by the token of their respectively different plant size and rate of water use. Thus, the effect of HVA1 on physiological factors ascribing drought tolerance must be explored in isolation of its effect on plant morphology. In the present study, both the transgenic and NT plants with normal growth and development are compared (Plate 1). In earlier studies, the transgenic rice lines expressing HVA1 protein did not manifest detrimental effects on the growth and development of the rice plants and were morphologically similar to NT plants [8]. Thus, the difference in plant water relations between transgenic and NT plants in the present study was primarily the effect of HVA1 protein and not due to any differences in plant growth among transgenic and NT lines.

The rice plants were grown in large pots and were subjected to a long but gradual drought stress cycle. The duration of the period from the last irrigation to wilting of the plants under drought stress varied between 28 and 42 days in NT and transgenic plants, respectively. The RWC, LWP and OP data taken the day after the last irrigation represented a well-watered (non-stress) state with no his-

Plate 1. Performance of transgenic (#30–59 and 30–54) and non-transgenic (Nipponbare) rice lines 25 days after withholding water.

tory of water deficit. There were no significant differences among the transgenic and NT lines for RWC, LWP and OP under non-stress condition. The non-stress RWC was 96.5, 96.8 and 97.0%, respectively in #30–59, 30–54 and NT rice lines (Fig. 1). The non-stress LWP was −1.10, −1.34 and −1.15 MPa, respectively in #30–59, 30–54 and NT lines (Fig. 2). The OP of the rice lines was −1.57, −1.68 and −1.67 MPa, respectively in #30–59, 30–54 and NT lines under non-stress condition (Fig. 3). Similar non-stress leaf RWC, LWP and OP were reported for rice [28,36]. There was no significant difference in relative membrane injury among transgenic and NT lines and the cell membrane leakage was 1.6, 1.1 and 2.0%, respectively in #30–59, 30–54 and NT lines under non-stress condition. Similar results were reported earlier for rice under non-stress conditions [37].

During the initial 14 days after withholding water, there was no significant decrease in plant water status, viz. RWC and LWP, either in the transgenic or NT rice lines. Rice plants maintained near turgid water status without symptoms of leaf rolling for more than 12 days after cessation of rain under field conditions in rainfed ecosystem [38]. Thus, the rice plants were subjected to a prolonged stress cycle in this study similar to field drought stress in the target production environment. The differences in plant water status between the transgenic and NT lines however, became evident during

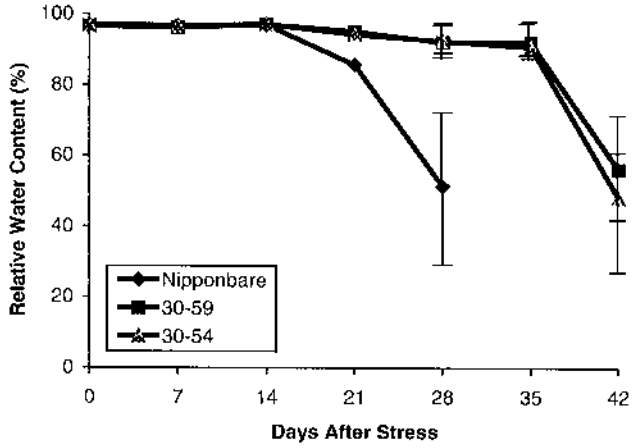

Fig. 1. Changes in leaf relative water content in non-transgenic and transgenic lines (#30–59 and 30–54) of rice (cv. Nipponbare) under progressive water stress.

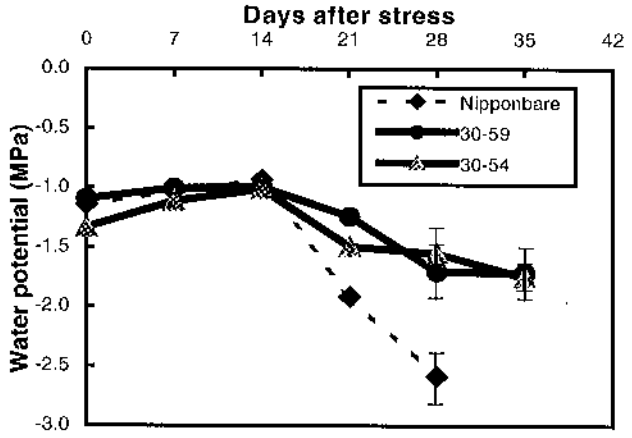

Fig. 2. Changes in leaf water potential in non-transgenic and transgenic lines (#30–59 and 30–54) of rice (cv. Nipponbare) under progressive water stress.

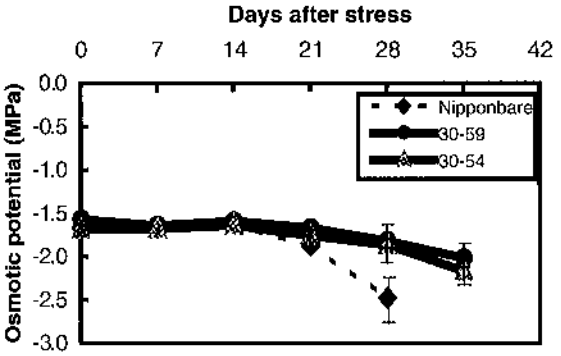

Fig. 3. Changes in leaf osmotic potential in non-transgenic and transgenic lines (#30–59 and 30–54) of rice (cv. Nipponbare) under progressive water stress.

the third week of the stress cycle. RWC declined to 85.6% in the NT line, while it was relatively higher in the transgenic lines (95.0 and 94.1%, respectively in #30–59 and 30–54) 21 days after stress (Fig. 1). LWP also declined considerably in NT plants (−1.92 MPa), whereas LWP was relatively higher in the transgenic lines (−1.25 and −1.50 MPa, respectively in #30–59 and 30–54) 21 days after withholding water (Fig. 2). There was a decrease in OP of NT plants (−1.88 MPa) as compared to transgenic lines (−1.67 and −1.76 MPa, respectively in #30–59 and 30–54) 21 days after stress (Fig. 3).

A set of plants in both transgenic and NT lines were maintained under well-watered (non-stress) condition with regular irrigation. Measurements of RWC, LWP, OP and cell membrane injury were made in this set of plants coinciding with similar measurements in stressed plants 21 days after withholding water. The values were comparable with those obtained on 40 DAS under non-stress conditions, the day after with holding water. For instance, RWC ranged from 96.5 to 97.2% among the three rice lines during 40–62 DAS under non-stress condition. LWP ranged between −0.94 and −1.34 MPa among the rice lines under non-stress condition over the growth period. Similar non-significant changes in LWP over crop growth period under non-stress conditions were reported for rice [39]. OP varied from −1.57 to −1.68 MPa among the rice lines under non-stress conditions. There was no significant difference in cell membrane leakage under non-stress condition due to shift in developmental stage and it varied from 1.11 to 2.04% among the different rice lines over the growth period. The results indicated that there was no significant changes in plant water status indicators, viz. RWC, LWP and OP due to change in developmental stages among the different rice lines under non-stress condition.

The differences in plant water status between transgenic and NT plants became well pronounced by 28 days after stress. At this time, RWC of NT plants was as low as 51.2%, whereas the transgenic lines maintained relatively higher RWC, 92.0 and 92.3%, respectively in line #30–59 and 30–54 (Fig. 1). Leaves normally desiccate and die when they reach a critical RWC [40]. Rice leaves loss turgor at 70% RWC [28] and rice plants wilt at RWC of 66% [41]. Thus, NT plants lost their turgor (−0.11 MPa), while the transgenic lines maintained a positive turgor potential (0.11 and 0.31 MPa, in line #30–59 and 30–54, respectively) 28 days after stress and leaves started rolling and drying earlier in NT plants (between 21 and 28 days after stress) as compared to transgenic lines. LWP was also lower in NT lines (−2.59 MPa) as compared to transgenic lines (−1.56 to −1.71 MPa, respectively in line #30–59 and 30–54), 28 days after withholding water (Fig. 2). LWP of −2.5 MPa is deleterious to rice production under field drought stress conditions [38]. OP was lower, −2.48 MPa in NT line 28 days after with holding water as compared to transgenic lines, −1.82 and −1.87 MPa, respectively in line #30–59 and 30–54 (Fig. 3). The decrease in OP of NT plants might be largely the result of concentration of cellular contents due to excessive loss of leaf moisture, as indicated by low RWC and LWP. NT plants had higher cell membrane injury (8.29%) as compared to transgenic lines, 0.54 and 0.71%, respectively in line #30–59 and 30–54, 28 days after stress (Fig. 4). The duration of the period from last irrigation to wilting of plants was 28 days for NT and 42 days for transgenic rice plants. There was no significant difference between transgenic and NT lines for OA, 0.48, 0.57 and 0.49 MPa, respectively in #30–59, 30–54 and NT lines (Fig. 5).

Limited water availability leads to reduced growth of aerial parts and, to a lesser extent, of the root system. The transgenic plants showed lesser reduction in shoot and root growth under drought stress as compared to NT plants. Plants under well-watered (non-stress) and stress conditions were sampled at the end of the experiment and shoot and root dry weights were determined. The NT plants showed 48.3 and 26.4% reduction in shoot and root dry weight, respectively under stress as compared to non-stress condition (Fig. 6). However, the transgenic lines showed lesser reduction in shoot (33.6 and 20.1%, respectively for line #30–59 and 30–54) and root (6.0 and 10.0%, respectively for line #30–59 and 30–54) dry weight under drought stress as compared to non-stress conditions.

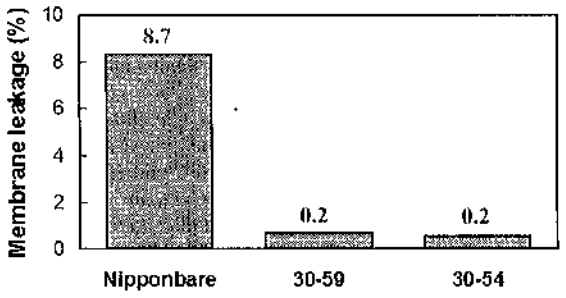

Fig. 4. Difference in cell membrane leakage among non-transgenic and transgenic lines (#30–59 and 30–54) of rice (cv. Nipponbare) 28 days after withholding water. The values above the bars are S.D. of the mean.

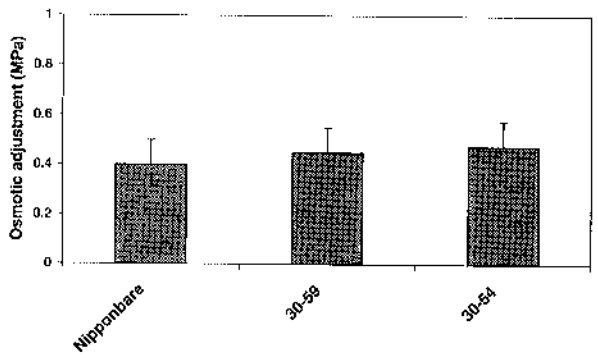

Fig. 5. Osmotic adjustment in non-transgenic and transgenic lines (#30–59 and 30–54) of rice (cv. Nipponbare) under prolonged water stress. Osmotic adjustment was determined as the difference in turgid leaf osmotic potential between non-stressed (well-watered) and stressed plants (RWC = ~ 60%) after rehydration (28). The stressed plants lost ~40% moisture from full turgor during the stress treatment.

The cell membrane is one of the main sites common to different stresses [42]. Drought induces membrane injury leading to metabolic dysfunction. The extent of its damage is used as a measure of tolerance of plants to various abiotic stresses such as drought [33] and salt [43]. Increased electrolyte leakage from damaged leaf tissues is generally considered an index of membrane damage and deterioration [44]. Transgenic plants had better cell membrane stability under drought stress as compared to NT plants. The results of the present study thus suggest that the improved drought resistance in these two transgenic lines might have been achieved via cell membrane protection. However, this has to be further verified by subjecting both NT and transgenic plants to severe but uniform level of tissue water stress.

OA is an important component of drought tolerance in crop plants. Generally, when cells are subjected to slow dehydration, compatible solutes accumulate in the cytosol resulting in the maintenance of cell water content against the reduction in apoplastic water potential [45]). This phenomenon is time-dependent [46]. Our earlier studies indicated that rice requires 3–4 weeks of continuous drought stress for good expression of potential OA [28]. In the present study, both transgenic and NT plants were subjected to a slow drying cycle over a period of 28–35 days. Despite this prolonged drying cycle, neither the transgenic nor the NT lines showed high levels of OA. There was no significant difference between transgenic and NT lines for OA and both had low levels of OA (0.48–0.57 MPa) under drought stress. The reasons for lack of OA in the rice lines are many fold. Rice ecotypes differ in their capacity for OA and japonica ecotypes, e.g. Nipponbare used in the present study, are in general known to have low OA capacity [28,36]. Further, the compatible solutes differ with plant species [47] and total soluble sugars and K^+ ions are found to be the major osmolytes contributing to OA under drought stress in rice in experiments done in this laboratory (Tripathy et al. personal communication). Thus, LEA proteins may not function as osmolytes in rice.

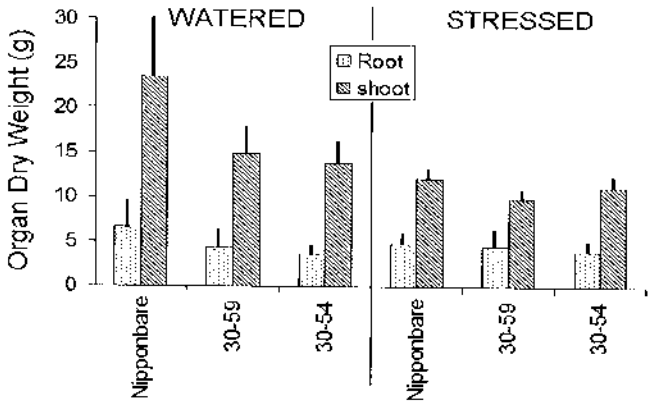

Fig. 6. Differences in shoot and root dry weight among non-transgenic and transgenic lines (#30–59 and 30–54) of rice (cv. Nipponbare) under well-watered and water-stress conditions.

Reports indicate a possible role of LEA proteins in dehydration tolerance, probably through maintenance of protein or membrane structure, sequestration of ions, binding of water, and operation as molecular chaperones [48,49]. LEA proteins accumulate in response to water deficit in many plants, and these are particularly abundant in anhydrobiotic plants such as the resurrection plant *Craterostigma plantagineum* and in maturing seeds and pollen [50]. The group-3 LEA motif has been proposed to form an amphipathic alpha helix that directs the oligomerization of the protein with possibilities for intra- and inter-molecular interactions [51]. These proteins are extremely hydrophilic and are resistant to denaturation by heat, prompting suggestion that they help to prevent damage by water stress. Three classes of LEA proteins have been genetically engineered and shown to provide dehydration tolerance in transgenic rice plants: PMA1959, a group 1 LEA protein and PMA80, a group 2 LEA protein both from wheat [16], and HVA1, a group 3 LEA protein from barley [8]. Transgenic wheat lines expressing *HVA1* gene had significantly higher water use efficiency than non-transformed lines [51]. Accumulation of LEA proteins was shown to confer salt tolerance in transgenic rice lines through better cell membrane protection [12,16]. Drought, salinity and freezing all cause cellular dehydration, thus the mode of action of these proteins may be common for different stresses.

In summary, plant drought tolerance is a complex trait that involves multiple physiological and biochemical mechanisms and regulation of numerous genes. A broad spectrum of genes is expressed on exposure to dehydration. Information about the physiological functions of these genes is essential for successful engineering of dehydration tolerance in crop plants. In this study, transgenic rice lines expressing barley *HVA1* gene were tested under prolonged drought stress cycle to understand the mechanism of dehydration tolerance. The results suggest that LEA proteins confer dehydration tolerance in transgenic rice plants not through OA but probably via cell membrane protection.

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