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ORIGINAL PAPER

# Over-expression of a *LEA* gene in rice improves drought resistance under the field conditions

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**Abstract** Late embryogenesis abundant (LEA) proteins have been implicated in many stress responses of plants. In this report, a LEA protein gene *OsLEA3-1* was identified and over-expressed in rice to test the drought resistance of transgenic lines under the field conditions. *OsLEA3-1* is induced by drought, salt and abscisic acid (ABA), but not by cold stress. The promoter of *OsLEA3-1* isolated from the upland rice IRAT109 exhibits strong activity under drought- and salt-stress conditions. Three expression constructs consisting of the full-length cDNA driven by the drought-inducible promoter of *OsLEA3-1* (OsLEA3-H), the *CaMV* 35S promoter (OsLEA3-S), and the rice *Actin1* promoter (OsLEA3-A) were transformed into the drought-sensitive *japonica* rice Zhonghua 11. Drought resistance pre-screening of $T_1$ families at anthesis stage revealed that the over-expressing families with OsLEA3-S and OsLEA3-H constructs had significantly higher relative yield (yield under drought stress treatment/yield under normal growth conditions) than the wild type under drought stress conditions, although a yield penalty existed in $T_1$ families under normal growth conditions. Nine homozygous families, exhibiting over-expression of a single-copy of the transgene and relatively low yield penalty in the $T_1$ generation, were tested in the field for drought resistance in the $T_2$ and $T_3$ generations and in the PVC pipes for drought tolerance in the $T_2$ generation. Except for two families (transformed with OsLEA3-A), all the other families (transformed with OsLEA3-S and OsLEA3-H constructs) had higher grain yield than the wild type under drought stress in both the field and the PVC pipes conditions. No significant yield penalty was detected for these $T_2$ and $T_3$ families. These results indicate that transgenic rice with significantly enhanced drought resistance and without yield penalty can be generated by over-expressing *OsLEA3-1* gene with appropriate promoters and following a bipartite (stress and non-stress) in-field screening protocol.

**Keywords** Abiotic stress · Grain yield · Promoter · *Oryza sativa*

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## Introduction

Continuous increase of world population, ever increasing deterioration of arable land, scarcity of fresh water, and global climate changes all underscore the importance of developing stress-resistant crops. Drought is one of the major abiotic stresses affecting plant growth and reducing crop productivity. It has been estimated that 70% of the crop yield loss can be attributed to abiotic stresses, especially drought (Bray et al. 2000). Due to the severe detrimental impact of drought on the crop yield, engineering drought resistant crops has become a challenging task for crop scientists. Conventional breeding of drought resistance has been a basic approach for a long time and some successes have been achieved in crops such as maize (Hoisington et al. 1996), rice (Zhang et al. 2006), and wheat (Zhao et al. 2000b). However, a big gap remains between the current resistance levels and what is needed for most of the major crops. This is especially true for rice because its yield stability is more sensitive to water scarcity than other upland crops.

In response to drought stress, plants have evolved mechanisms to perceive and transmit the stress signals to cellular machinery that activate adaptive responses (Thomashow 1999; Xiong et al. 2002). Drought resistance is a complex trait that is influenced by coordinated expression of a network of genes (Thomashow 1999; Xiong et al. 2002) and affected by a large number of environmental, anatomical, physiological, biophysical, biochemical and developmental factors (Soltis and Soltis 2003), making progress in genetic improvement of drought resistance quite slow. The rapid development of functional genomics and biotechnology in last decade has provided new opportunities in improving drought resistance. To date, quite a few reports suggest that increased expression of drought stress related genes could improve drought resistance to some extent in important crops (Xu et al. 1996; Zhang et al. 2001; Garg et al. 2002; Hu et al. 2006).

One efficient strategy for improving drought resistance of plants is to increase the content of soluble sugars and other compatible solutes through transgenic approaches. These compounds, such as proline, trehalose, betaine and mannitol, serve as osmoprotectants and, in some cases, stabilize functional molecules under stress conditions (Kishor et al. 1995; Hayashi et al. 1997; Shen et al. 1997; Garg et al. 2002). Late embryogenesis abundant (LEA) proteins have also been implicated in water deficit stress (Xu et al. 1996; Maqbool et al. 2002; Goyal et al. 2005). LEA proteins have been classified into five major groups based on amino acid sequences (Bake et al. 1988; Dure et al. 1989) and were re-examined recently using statistically based bioinformatics tools (Wise 2003). These proteins are part of evolutionarily conserved group of hydrophilic proteins termed "hydrophilins" involved in various adaptive responses to hyperosmotic conditions (Garay-Arroyo et al. 2000). The majority of LEA proteins display a preponderance of hydrophilic and charged amino acid residues. Expression of *LEA* genes, which often appears to be abscisic acid-dependent, was detected not only in seeds but also in vegetative tissues with water deficit associated with drought, salt, and cold stresses (Ingram and Bartels 1996; Thomashow 1998; Cuming 1999; Grelet et al. 2005). Both the pattern of expression and the structural features of LEA proteins suggest a general protective role in desiccation tolerance (Ingram and Bartels 1996). Despite massive data on the expression and protein structure (Raynal et al. 1999; NDong et al. 2002; Grelet et al. 2005), little work has been reported on the manipulation of *LEA* genes to improve drought resistance under field conditions. For example, the *HVA1* gene encoding group 3 LEA protein from barley (*Hordeum vulgare* L.) was transformed into rice and the tolerance to water deficit and salt stress of the transgenic rice was improved under the greenhouse conditions (Xu et al. 1996).

In this study, a full-length cDNA clone of a drought and salt stress-responsive *LEA* gene, *OsLEA3-1*, was transformed into rice in order to assess the effect of its expression under the control of three different promoters on improving drought resistance under the field conditions. Our results indicate that drought resistance is significantly improved in the transgenic rice with expression of the *OsLEA3-1* transgene controlled by a drought-inducible *HVA1-like* promoter and a constitutive promoter *CaMV* 35S.

## Materials and methods

### Isolation, construction and transformation of *OsLEA3-1*

In our previous studies on drought-stressed expression profiles in rice seedlings using a cDNA microarray containing about 9,000 unique expressed sequence tags (ESTs) (unpublished data), a cDNA gene showed strong induction by drought stress in the upland rice IRAT109 (*Oryza sativa* L. ssp *japonica*). The full-length cDNA of this gene, designated *OsLEA3-1*, was identified in the cDNA library of the *indica* rice Minghui 63 constructed by Chu et al (2003).

Three binary expression constructs (OsLEA3-S, OsLEA3-A, and OsLEA3-H) were generated by inserting the full-length cDNA (released from the cDNA vector pSPORT1 by *Bam*HI and *Kpn*I) into backbone vectors pCAMBIA1301S, pCAMBIA1301A, and pCAMBIA1301H, respectively. These backbone vectors were developed by inserting a double *CaMV* 35S, rice *Actin1* and *HVA1-like* promoter, respectively, into the multiple cloning sites (*Hin*dIII and *Sac*I) of pCAMBIA1301 (provided by the Center for the Application of Molecular Biology for International Agriculture, Australia). The *HVA1-like* promoter was isolated from the upland rice IRAT109 based on the genomic sequence of *OsLEA3-1* promoter in rice cultivar Nipponbare using *Hin*dIII-adapted primer 5′-TCC*AAGCTT*AAGGGCCTCCATAACCTACG-3′ and *Sac*I-adapted primer 5′-TCG*GAGCTC*ACGCGCGAATGTTAGAACTC-3′. The *HVA1-like* promoter was also amplified by *Hin*dIII-adapted primer (5′-TCC*AAGCTT*GATCTGTGGTGATCGACTTG-3′) and *Bam*HI-adapted primer (5′-TCG*GGATCC*ACGCGCGAATGTTAGAACTC-3′) and fused to the *GUS* reporter gene in the vector pCAMBIA1391Z (named 1391-H) for assessing the stress-induced activity of the promoter.

All the expression vectors were introduced into *japonica* rice Zhonghua 11 (drought-sensitive) by *Agrobacterium*-mediated transformation (Hiei et al. 1994; Lin and Zhang 2005). The embryonic calli from Zhonghua 11 seeds were cultured for 3 days at 28°C with the *Agrobacterium* strain *EHA105* that carried the cDNA or promoter constructs and then transferred to the selection medium containing 50 μg/ml

hygromycin and 200 μg/ml carbenicillin. After 2–3 cycles (2 weeks per cycle) of selection, resistant calli were transferred to the pre-regeneration medium containing 40 μg/ml hygromycin. After 7 days, the resistant calli were transferred to the regeneration medium without hygromycin to regenerate plantlets.

### PCR, southern and RNA gel blot analysis

Genomic DNA was extracted by using the CTAB method (Zhang et al. 1992). The hygromycin phosphotransferase (*Hpt*) gene-specific primers (5′-AGAAGAAGATGTTGG CGACCT-3′ and 5′-GTCCTGCGGGTAAATAGCTG-3′) were used to identify positive transgenic plants. PCR reaction was conducted in a volume of 20 μl containing 100 ng genomic DNA, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1× PCR buffer, 0.2 μM of each primer, and 1 unit *rTaq* Polymerase (TaKaRa, Dalian, China). The PCR reaction was performed at 94°C for 5 min; then with 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; finally at 72°C for 5 min.

Copy number of the transgene was determined by Southern blot analysis using the *Hpt* gene as a probe (amplified by the two *Hpt*-specific primers). Three micrograms of genomic DNA from each sample was digested with *Eco*RI, fractionated on 0.7% agarose gel, and blotted onto nylon membranes, which were hybridized with a $^{32}$P-dCTP-labeled *Hpt*-specific probe using standard protocols (Sambrook et al. 1989).

Total RNA samples analyzed in this study were isolated from leaf tissues using TRIzol reagent (Life Technologies, Rockville, MD, USA). Mixed leaf tissues from normal growing (for OsLEA3-S and OsLEA3-A constructs) or drought-stressed (leaf rolled, for OsLEA3-H construct) transgenic $T_1$ families were used for the expression identification of *OsLEA3-1*. Fifteen micrograms of total RNA from each sample were separated on 1.2% agarose gel containing formaldehyde and then transferred onto a nylon membrane, and hybridized with the $^{32}$P-labeled *OsLEA3-1* gene-specific fragment obtained by digestion of the cDNA plasmid of this gene with *Bam*HI and *Kpn*I. Hybridization and washing conditions were based on standard protocols (Sambrook et al. 1989).

### GUS activity assay of transgenic plants

GUS assay was performed using the standard protocol (Jefferson et al. 1987). Histochemical staining for GUS expression was performed essentially as described by Wu et al. (2003). Rice tissues were incubated in the GUS staining solution (50 mM sodium phosphate at pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mg/ml of X-Gluc, 100 μg/ml of chloramphenicol, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, and 20% methanol), placed under a mild vacuum for 10 min, and then incubated at 37°C for about 36 h. After removing staining solution, tissues were fixed (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) and examined.

### Abiotic stress treatments at seedling stage

Abiotic stress and ABA treatments were conducted mainly according to Xiong and Yang (2003). The 3-week old seedlings of *japonica* rice IRAT109 were prepared by growing plants in plastic trays filled with sandy soil in the greenhouse with a 14-h light/10-h dark cycle at 28°C. Drought stress was conducted by withholding water from the trays, and seedling leaves were sampled at 1, 2, 4, 6, 7, 8 days after drought stress treatment. For salt stress, seedling roots were immersed in 200 mM NaCl solution, and seedling leaves were sampled at 1, 3, 6, 18, 36, 72 h after stress treatment. In ABA treatment, seedling leaves were sprayed with 100 μM ABA and sampled at 1, 3, 6, 24, 48 h after treatment. For cold stress, seedlings were transferred to a growth chamber at 4°C, and sampled at 1, 3, 6, 24, 48, 72 h after treatment.

### Drought resistance testing of transgenic rice in the field

Thirty PCR-positive (checked at $T_0$ generation) $T_1$ families per construct were pre-screened for drought resistance in an isolated field equipped with a movable rain-off shelter (referred to shelter hereafter) located on the campus of Huazhong Agricultural University in 2004. Twenty plants from each family were planted in two rows (one plot). The wild type (WT) Zhonghua 11 was inserted after every five transgenic families for comparison. One month after transplanting, soil water was discharged through the outlets located 1.5 m below the top of concrete walls surrounding the field. Severe drought stress (soil water content was about 18%) occurred at flowering stage for the cultivar Zhonghua 11 grown in this field. Normal irrigation was resumed after pollination stage. A duplicate set of materials was planted in another isolated field with full irrigation to evaluate the difference of yield between the transgenic and WT rice.

Transgenic families for drought testing in the next generation were selected based on following criteria: no obvious morphological changes, lower yield penalty, increased drought resistance in terms of relative yield (yield under drought stress treatment/yield under normal growth conditions; Yue et al. 2006), over-expression (or drought-induced over-expression for OsLEA3-H) of a single copy transgene. For families satisfying such criteria, seeds from the plants with good drought resistance were harvested individually for testing in the $T_2$ generation. To identify homozygous families for drought resistance testing, root segments of 7-day old $T_2$ seedling were subjected to GUS assay. Only those families with more than

95% of GUS-positive seedlings (100 seedlings tested for each $T_2$ family) were considered to be homozygous lines and used for following drought resistance testing. Homozygous $T_2$ lines were tested under two drought stress treatments (sheltered field and PVC pipes), and control with normal-irrigation. Under the field conditions equipped with a movable shelter, selected homozygous lines and the wild type Zhonghua 11 were tested using a randomized complete block design with three replications. Each plot had 20 plants planted in two rows for each family with a space of 0.17 m between plants and 0.3 m between plots. Drought stress was applied as described above in the $T_1$ generation. The same design was used for the normal irrigation treatment. The planting and drought treatment in the PVC pipes (1 m in length and 0.2 m in diameter) was essentially the same as described previously (Yue et al. 2006). For each homozygous $T_2$ line, 20 plants were divided into two groups for drought stress and normal growth treatments, respectively, and planted individually in the PVC pipes placed under plastic tents (length × width × height; 26 × 6 × 3.6 m) with foldable roofs. The wild type plants were inserted after every ten transgenic plants for comparison. Drought stress was initiated at panicle development stage (ca. 2 weeks before flowering) by discharging water through a hole near the bottom of the pipes. Each plant was stressed to the same degree at which leaves of main tillers were completely rolled (observed at 6:00 p.m.), then irrigated thoroughly overnight and immediately subjected to another round of stress until complete leaf-rolling. After two rounds of drought stress, plants were irrigated to allow recovery at flowering and seed maturation stages.

Seeds were harvested from the homozygous $T_2$ lines and drought resistance testing was conducted in the $T_3$ generation in the field by following the same experimental design and stress treatment as in the $T_2$ generation.

### Data collection and statistical analysis

Grain yield per plant and spikelet fertility were used as the major criteria to evaluate the drought resistance performance of transgenic plants. For each $T_1$ family or homozygous line ($T_2$, $T_3$) in the field, yield and spikelet fertility of 16 plants from each plot (excluding 4 plants, 2 on each side of the plot) were measured, and the mean value of the 16 plants in each plot was used for statistical analysis. Relative yield was the ratio of yield (the mean value of the 16 plants in each $T_1$ family) under drought stress treatment to the yield under normal growth treatment (Yue et al. 2006). For the testing in the PVC pipes, yield and spikelet fertility of all the plants were individually measured, and the yield and spikelet fertility values of each plant under drought and normal growth conditions were used for statistical analysis.

The data on grain yield per plant, spikelet fertility, and relative yield were analyzed by one-way analysis of variance (ANOVA). The subsequent multiple comparisons among the means of transgenic families or lines and WT were examined based on the least significant difference (LSD) test. All statistical analysis was performed using SPSS package (version 12.0).

## Results

### Isolation and stress-induced expression of *OsLEA3-1*

We originally observed drought stress induction of *OsLEA3-1* using a rice cDNA microarray (unpublished data). A full-length cDNA clone was identified for this gene, which encodes a predicted protein of 200 amino acids belonging to the group 3 LEA family in a cDNA library of the *indica* rice Minghui 63 (Chu et al. 2003). OsLEA3-1 protein sequence has 97% identity to OsLEA3 identified previously in rice (Moons et al. 1997) and *OsLEA3-1* is considered to be an allele of *OsLEA3* based on its location in the rice genome. The OsLEA3-1 protein has 56% identity to HVA1 from barley (Hong et al. 1988), 52% identity to group 3 LEA protein MGL3 from maize, and 53% identity to group 3 LEA protein pMA2005 from wheat (Curry et al. 1991; Curry and Walker-Simmons 1993) as revealed by ClustalW (Chenna et al. 2003) analysis (Fig. 1).

RNA gel blot analysis was performed to investigate the expression of *OsLEA3-1* in the seedling leaves of upland rice IRAT109 treated by drought, salt, ABA and cold (Fig. 2a). Transcript level of *OsLEA3-1* began to increase at 6 days after water withholding and was strongly induced at 8 days after water withholding. After salt (200 mM NaCl) treatment, the *OsLEA3-1* transcript was induced within 18 h and peaked at 72 h. The *OsLEA3-1* was induced within 3 h after ABA treatment and its transcript level peaked at 48 h. However, the expression of *OsLEA3-1* was not induced by low temperature (4°C). These results indicated that *OsLEA3-1* was strongly induced by drought and salt stresses and ABA treatment but not by cold stress in the upland rice cultivar IRAT109.

### Identification of *OsLEA3-1* promoter

Since *OsLEA3-1* was strongly induced by drought in upland rice IRAT109, we isolated the promoter region (1,253 bp upstream of the transcribed sequence of the gene, designated *HVA1-like* promoter, accession no. DQ837728) from IRAT109 based on the genomic sequence of Nipponbare. Promoter sequence search against the PLACE database (http://www.dna.affrc.go.jp/PLACE/) suggested that more number of putative ABA responsive elements (ABRE) exist

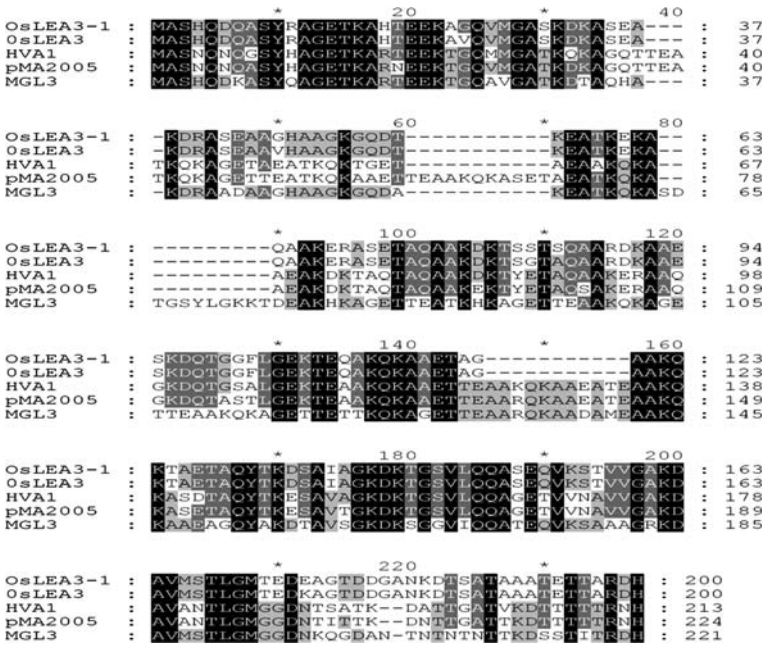


**Fig. 1** Alignment of deduced amino acid sequence of OsLEA3-1 with representative reported LEA proteins using ClustalW program (Chenna et al. 2003). *Dashes* were introduced to maximize sequence alignments. Identical (*black*) and conserved residues (*grey*) are highlighted. The accession numbers for the aligned sequences are as follows: *OsLEA3-1*, DQ789359; *OsLEA3*, AAV67829; *HVA1*, CAA31853; *MGL3*, CAA82632; *pMA2005*, CAA40204

in the promoter sequence from IRAT109 than in Nipponbare (data not shown). The *GUS* reporter gene under the control of the *HVA1-like* promoter from IRAT109 was transformed into rice Zhonghua 11. GUS activity was strongly induced by drought and salt stresses and ABA treatment but not by cold in the transgenic plants (Fig. 2b), which agrees well with the results from RNA gel blot analysis of *OsLEA3-1*. This promoter was therefore used for making the stress-inducible cDNA construct used in this study.

## Generation and identification of transgenic rice of *OsLEA3-1*

Three constructs, OsLEA3-S, OsLEA3-A and OsLEA3-H, were generated by fusing the cDNA of the *OsLEA3-1* gene with *CaMV* 35S, *Actin1* and *HVA1-like* promoter in the backbone vectors pCAMBIA1301S, pCAMBIA1301A, and pCAMBIA1301H, respectively (Fig. 3a). These constructs contain the *GUS* reporter gene under the control of *CaMV* 35S promoter, and the *Hpt* selection gene under the control of *CaMV* 35S promoter (Fig. 3a). Constructs were transformed into *japonica* rice Zhonghua 11, which is drought-sensitive compared to IRAT109 (unpublished data) and has a relatively high efficiency of transformation in comparison with *indica* rice cultivars (Lin et al. 2005). More than 600 transgenic plants, about 200 plants per construct, were generated. Transformed plants ($T_0$ generation) were identified by PCR using primers specific to the hygromycin resistance gene (*Hpt*). Among 204 independent regenerants checked, an expected band (564 bp) was amplified in 187, suggesting that more than 90% of regenerants were transformed (Fig. 3b–d).

Southern blot hybridization was performed using the *Hpt*-specific fragment as a probe. Among 90 independent transformants (30 plants per construct, Fig. 3b–d) checked, about 45% of the checked transformants harbored single copy of the transgene, and approximately 40% of the transformants contained 2–3 copies. These percentages are similar to previous reports (Garg et al. 2002; Wu et al. 2003). There were very few plants that appeared to be positive by PCR analysis but not positive by Southern hybridization. The hybridization pattern of each transgenic plant was unique, suggesting that these plants were derived from independent transformation events.

The expression level of *OsLEA3-1* in the mixed leaf tissues of 20 plants from each $T_1$ family derived from the $T_0$ transgenic plants analyzed by Southern hybridization was checked by RNA gel blot analysis using the *OsLEA3-1* gene as a probe (Fig. 3b–d). The percentage of over-expression (or drought-induced over-expression for OsLEA3-H) was 63, 56, and 46% for OsLEA3-S, OsLEA3-A, and OsLEA3-H, respectively. The transcript level of *OsLEA3-1* in the wild type (WT) plants was almost undetectable under drought stress conditions (Fig. 3b).

## Drought resistance pre-screening of $T_1$ transgenic families

$T_1$ families over-expressing the *OsLEA3-1* gene from each construct (Fig. 3b–d) were pre-screened for drought resistance under the sheltered field (drought stress was applied at anthesis stage) in 2004. Morphology and yield comparison under normal irrigation conditions were also examined at that time. Under the normal growth conditions, the average values of grain yield and spikelet fertility of $T_1$ families

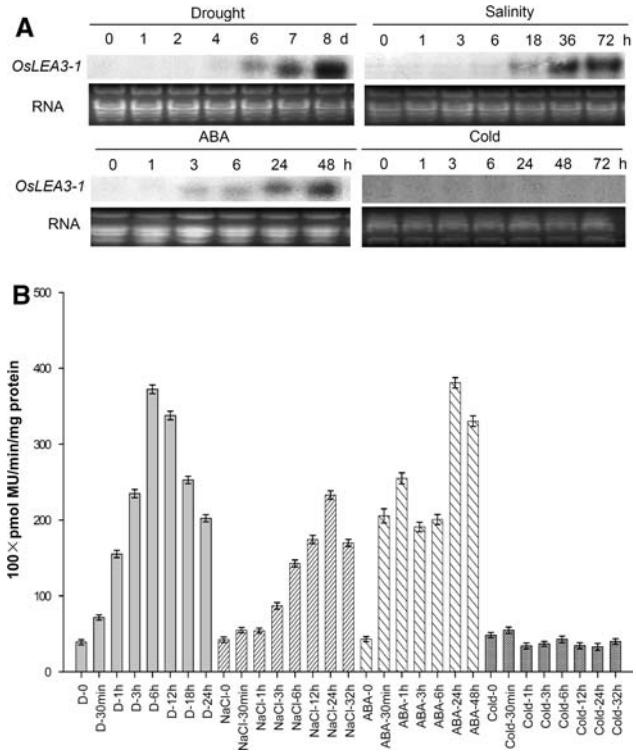


**Fig. 2** Stress-inducible expression of *OsLEA3-1* gene. **a** RNA gel blot analysis of *OsLEA3-1* expression in 3-week old IRAT109 seedlings treated with drought, 200 mM NaCl, 100 μM ABA, and cold. Total RNA was isolated from leaf tissues at the time points specified on the top of the blots (*d* days, *h* hours). The relative amount of total RNA loaded in *each lane* is shown by ethidium bromide staining. The probe is the cDNA of *OsLEA3-1* obtained by the digestion of the cDNA clone with *Bam*HI and *Kpn*I. **b** GUS activity assay of *OsLEA3-1* promoter::*GUS* in 3-week old transgenic plants treated by drought stress (with water deprived from the hydroponic cultured plants), salt (200 mM NaCl), ABA (100 μM) and cold (4°C) stress. Values are mean of three replications

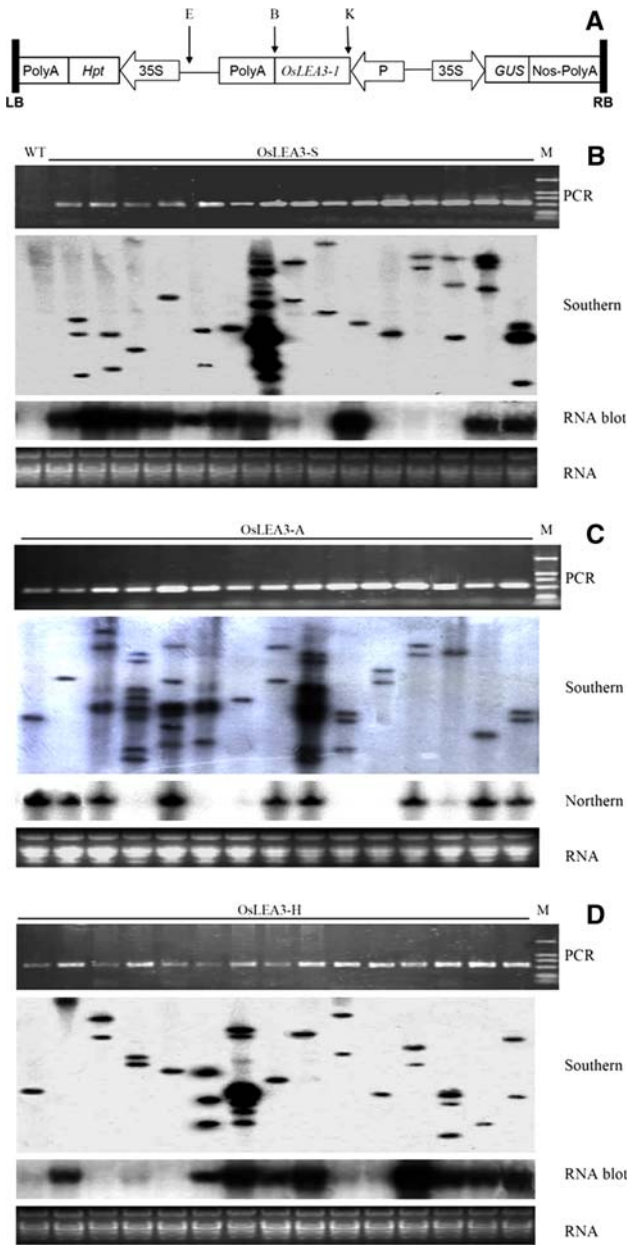


**Fig. 3** Molecular identification of transgenic plants. **a** Schematic diagram of the constructs for rice transformation. *P* represents *CaMV* 35S (*S*), *Actin1* (A), or *HVA1-like* promoter (H). *LB* and *RB* represent T-DNA *left* and *right* border, respectively. The hygromycin phosphotransferase gene (*Hpt*) under the control of *CaMV* 35S promoter was used as a selective marker gene. E: *Eco*RI; B: *Bam*HI; K: *Kpn*I. **b**–**d** PCR (*top* in each panel), Southern (*middle*) and RNA blot (*bottom*) analysis of transgenic plants of OsLEA3-S (**b**), OsLEA3-A (**c**), and OsLEA3-H (**d**) constructs. A total of 120 plants were analyzed and only 15 plants for each construct were shown. PCR was conducted using *Hpt* gene-specific primers. The *Hpt* and *OsLEA3-1* gene fragments were used as probes for Southern and RNA blot hybridization, respectively. *M*, 2 kb DNA marker; *WT*, wild type Zhonghua 11

over-expressing the *OsLEA3-1* gene for each construct (referred to over-expression group hereafter) were significantly ($P < 0.01$) lower than WT (Table 1). Only about 15% of transgenic families showed no significant difference in yield per plant compared to WT. The reduced yield of the transgenic plants under normal growth conditions (yield penalty) was mainly due to the reduced spikelet fertility (Table 1) as the number of spikelets per plant and the grain weight showed no significant difference between over-expression groups and WT (data not shown). Considering the yield penalty in $T_1$ transgenic families, the relative yield (ratio of the yield in the stressed field to that in the normal irrigation field), a reliable parameter for evaluation of drought resistance according to Yue et al. (2006), was compared between each over-expression group and WT (Table 1). The results suggested that the relative yield of over-expression groups of the OsLEA3-S (52.34%) and OsLEA3-H (54.31%) constructs were significantly ($P < 0.01$) higher than that of WT (36.42%). However, there was no significant difference in the relative yield between the over-expression group of OsLEA3-A construct (35.73%) and WT. We also observed segregation of drought sensitivity (such as leaf rolling, Fig. 4a–b) for

**Table 1** Grain yield and spikelet fertility of transgenic family groups ($T_1$) over-expressing *OsLEA3-1* under normal growth and drought stress conditions in 2004

| Construct | Spikelet fertility (%) | | Grain yield per plant (g) | | Relative yield (%)[a] |
|---|---|---|---|---|---|
| | Normal growth | Drought stress | Normal growth | Drought stress | |
| OsLEA3-S | 55.87 ± 3.71** | 37.85 ± 3.15 | 20.63 ± 1.04** | 10.53 ± 0.81 | 52.34 ± 4.62** |
| OsLEA3-A | 53.55 ± 1.58** | 16.83 ± 1.27 | 19.41 ± 1.42** | 5.52 ± 0.48 | 35.73 ± 4.16 |
| OsLEA3-H | 56.21 ± 2.51** | 36.83 ± 2.17 | 21.23 ± 2.03** | 11.58 ± 1.26 | 54.31 ± 3.86** |
| WT | 76.67 ± 2.97 | 41.46 ± 2.41 | 32.55 ± 1.01 | 11.67 ± 0.58 | 36.42 ± 1.52 |

Values are mean ± SE ($n$ = 18, the number of over-expressed families in pre-screening for the OsLEA3-S construct; $n$ = 16 for the OsLEA3-A construct; $n$ = 14 for the OsLEA3-H construct; $n$ = 20 for WT)

[a] Value of relative yield means the ratio of the yield in stress to that in normal growth. Statistical analysis was performed between each over-expression group of the three constructs and WT by one-way ANOVA followed by the LSD test

** Significant difference between transgenic families groups and WT at the probability level of $P < 0.01$ by the LSD test

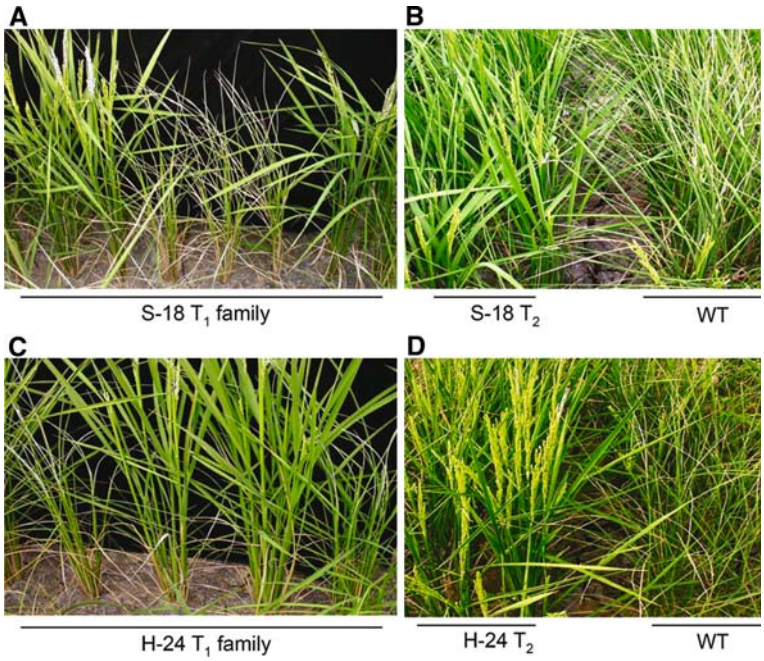


**Fig. 4** Drought resistance of transgenic families in the field. Photographs were taken just before re-watering for recovery after the drought stress at the anthesis stage in the field. **a–b** Segregation of drought resistance (degree of leaf-rolling) within $T_1$ transgenic families over-expressing a single copy of the transgene with OsLEA3-S (**a**) and OsLEA3-H (**b**) constructs. **c–d** Field performance of homozygous $T_2$ lines with OsLEA3-S (**c**) and OsLEA3-H (**d**) constructs under drought stress conditions. *WT* wild type Zhonghua 11

some $T_1$ families over-expressing a single copy of the transgene. These results suggested that expression of *OsLEA3-1* by a drought-inducible *HVA1-like* promoter and constitutive promoter *CaMV* 35S had significant effect on improving drought resistance in terms of relative yield, though a severe yield penalty existed in the $T_1$ generation.

## Drought resistance testing of homozygous $T_2$ lines

To confirm the increased drought resistance of the $T_1$ families, $T_2$ seeds from the transgenic plants showing improved drought resistance within the $T_1$ families were harvested to identify homozygous lines by GUS staining. The $T_1$ families from which $T_2$ seeds were harvested contained single copies of the transgenes which were over-expressed constitutively (OsLEA3-S) or during drought (OsLEA3-H) and exhibited relatively lower yield penalty. Three OsLEA3-S (S-6, S-18, S-21), two OsLEA3-A (A-2, A-29), and four OsLEA3-H (H-8, H-14, H-23, H-24) homozygous $T_2$ lines were selected for drought resistance testing in the sheltered field and PVC pipes in 2005.

In the field with normal irrigation, grain yield per plant of all the transgenic lines (28.5–30.8 g) and WT (31.1 g) was not significantly different (Table 2), suggesting very little yield penalty, if any, in these lines. Based on this result, grain yield was used for comparing drought resistance between these transgenic lines and WT. Under severe drought-stressed field conditions, the homozygous transgenic lines with OsLEA3-S and OsLEA3-H constructs showed less rolled or dead leaves than WT at the flowering stage (Fig. 4c–d). The grain yield per plant of all seven homozygous lines with the OsLEA3-S and OsLEA3-H

**Table 2** Grain yield and spikelet fertility of homozygous $T_2$ lines under normal growth and drought stress conditions in the sheltered field and PVC pipes (Wuhan, China, 2005)

| Construct | Homozygous $T_2$ line | Grain yield per plant (g) | | | | Spikelet fertility (%) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Field | | PVC pipes | | Field | | PVC pipes | |
| | | Normal growth | Drought stress | Normal growth | Drought stress | Normal growth | Drought stress | Normal growth | Drought stress |
| OsLEA3-S | S-6 | 28.45 ± 1.29 | 15.55 ± 1.32 | 31.25 ± 1.63 | 20.61 ± 1.24 | 80.3 ± 3.6 | 45.7 ± 2.1* | 79.2 ± 2.5 | 59.3 ± 3.2 |
| | S-18 | 29.05 ± 2.42 | 17.72 ± 1.56* | 31.96 ± 2.02 | 21.74 ± 1.43* | 79.4 ± 3.5 | 48.2 ± 1.6** | 81.3 ± 3.4 | 66.2 ± 3.5** |
| | S-21 | 28.84 ± 1.95 | 15.24 ± 1.69 | 31.53 ± 2.35 | 20.43 ± 1.08 | 77.4 ± 2.6 | 42.9 ± 1.8 | 79.3 ± 2.6 | 57.2 ± 2.9 |
| OsLEA3-A | A-2 | 28.98 ± 2.65 | 12.38 ± 2.03 | 31.22 ± 1.85 | 17.88 ± 0.78 | 78.3 ± 3.1 | 37.7 ± 1.5 | 77.4 ± 3.2 | 53.5 ± 3.6 |
| | A-29 | 28.63 ± 2.58 | 12.02 ± 1.32 | 31.08 ± 2.04 | 17.47 ± 0.93 | 81.5 ± 2.7 | 38.2 ± 2.6 | 77.9 ± 3.4 | 54.6 ± 2.6 |
| OsLEA3-H | H-8 | 30.29 ± 1.79 | 17.84 ± 1.49* | 32.94 ± 2.31 | 21.82 ± 0.89* | 77.2 ± 2.1 | 49.1 ± 2.7** | 78.5 ± 2.7 | 66.7 ± 2.8** |
| | H-14 | 28.57 ± 2.86 | 15.67 ± 1.38 | 32.73 ± 1.53 | 21.34 ± 1.09 | 77.5 ± 2.2 | 46.5 ± 2.2* | 80.4 ± 3.8 | 63.8 ± 2.7* |
| | H-23 | 29.16 ± 2.54 | 15.71 ± 1.22 | 32.86 ± 1.82 | 21.36 ± 2.06 | 81.8 ± 3.7 | 44.2 ± 2.7 | 81.3 ± 3.6 | 56.4 ± 3.8 |
| | H-24 | 30.81 ± 1.48 | 17.78 ± 1.62* | 33.25 ± 1.74 | 23.58 ± 1.58** | 79.6 ± 3.6 | 48.3 ± 3.3** | 81.6 ± 2.8 | 67.3 ± 3.2** |
| CK | WT | 31.07 ± 1.08 | 12.13 ± 0.97 | 33.57 ± 1.84 | 17.77 ± 1.36 | 81.2 ± 2.7 | 38.6 ± 1.5 | 82.3 ± 2.1 | 51.9 ± 1.8 |

Values are mean ± SE ($n = 3$ plots per line in sheltered field conditions or $n = 10$ plants per line in PVC pipes conditions). Data on grain yield per plant and spikelet fertility of homozygous $T_2$ lines and WT were analyzed by one-way ANOVA test, and the difference between each transgenic line and WT was examined by the LSD test

*, ** Significant differences between the means of transgenic lines and WT at the levels of $\alpha = 0.05$ and $\alpha = 0.01$, respectively

constructs (15.2–17.8 g) was higher or significantly ($P < 0.05$ for S-18, H-8, H-24) higher than that of WT (12.1 g), whereas the two lines (A-2, A-29) with OsLEA3-A construct (12.0–12.4 g) showed no difference compared to WT. These transgenic lines were also tested for drought tolerance, a major mechanism for drought resistance, by growing the plants in PVC pipes according to Yue et al. (2006). Except for A-2 and A-29 lines, grain yield of all the other seven lines (20.4–23.6 g per plant) was higher or significantly ($P < 0.05$ for S-18, H-8, H-24) higher than that of WT (17.8 g per plant) under drought stress conditions (Table 2). Under normal irrigation conditions, all the lines and WT showed no significant difference in yield (Table 2).

In addition to grain yield, spikelet fertility (%) of the seven homozygous $T_2$ lines for OsLEA3-S and OsLEA3-H constructs was higher than WT under drought stress conditions in both the field and the PVC pipes. Significantly ($P < 0.05$) higher spikelet fertility than WT was observed in five lines (S-6, S-18, H-8, H-14, H-24) in the sheltered field experiment and four lines (S-18, H-8, H-14, H-24) in the PVC pipes experiment. No significant difference was detected between any transgenic lines and WT under the normal irrigation conditions (Table 2). Moreover, no significant difference was detected for the other two yield component traits (number of spikelets per plant and grain weight) between transgenic lines and WT in all treatments.

## Drought resistance testing of homozygous $T_3$ lines

To verify the improved drought resistance of the $T_2$ lines described above, nine homozygous $T_3$ lines derived from the nine $T_2$ lines tested in 2005 were tested under both drought stress and normal growth conditions in the field in 2006. The results showed that six $T_3$ lines (S-6, S-18, H-8, H-14, H-23, H-24) with the OsLEA3-S and OsLEA3-H constructs had significantly ($P < 0.05$) higher grain yield per plant than WT under the drought stress conditions, and the spikelet fertility of all the homozygous $T_3$ lines with OsLEA3-S and OsLEA3-H constructs was significantly ($P < 0.05$) higher than that of WT (Table 3). No significant difference in grain yield and spikelet fertility was detected between these $T_3$ lines and WT under normal growth conditions (Table 3). For the A-2 and A-29 lines containing the OsLEA3-A construct, the yield and spikelet fertility were not significantly ($P < 0.05$) different from those of WT under drought stress conditions.

Two other yield components (number of spikelets per plant and grain weight) were also investigated under both drought stress and normal field conditions. In both cases, no significant difference was detected between the $T_3$ lines and WT, suggesting that the better yield performance of transgenics under drought stress conditions was mainly due to the relatively higher spikelet fertility.

**Table 3** Grain yield and spikelet fertility of homozygous $T_3$ lines under normal growth and drought stress conditions in sheltered field (Wuhan, China, 2006)

| Construct | Homozygous $T_3$ line | Grain yield per plant (g) | | Spikelet fertility (%) | |
|---|---|---|---|---|---|
| | | Normal growth | Drought stress | Normal growth | Drought stress |
| OsLEA3-S | S-6 | 29.42 ± 1.32 | 17.04 ± 1.02* | 82.5 ± 3.5 | 47.7 ± 2.5* |
| | S-18 | 28.78 ± 1.06 | 17.65 ± 1.45* | 79.8 ± 3.7 | 51.3 ± 3.3** |
| | S-21 | 29.45 ± 1.63 | 15.35 ± 1.55 | 79.2 ± 3.2 | 47.3 ± 2.8* |
| OsLEA3-A | A-2 | 29.46 ± 1.75 | 13.12 ± 1.52 | 79.7 ± 3.4 | 41.6 ± 3.0 |
| | A-29 | 29.34 ± 1.35 | 13.28 ± 1.53 | 80.2 ± 2.9 | 42.2 ± 3.1 |
| OsLEA3-H | H-8 | 29.89 ± 1.42 | 18.87 ± 1.57** | 79.2 ± 3.1 | 52.6 ± 3.8** |
| | H-14 | 29.42 ± 1.87 | 17.42 ± 1.43* | 80.3 ± 3.4 | 48.7 ± 3.4* |
| | H-23 | 30.16 ± 1.85 | 17.16 ± 1.71* | 79.6 ± 2.5 | 47.4 ± 2.2* |
| | H-24 | 29.38 ± 1.84 | 18.22 ± 2.16* | 79.8 ± 3.3 | 52.4 ± 3.3** |
| CK | WT | 31.14 ± 0.98 | 12.08 ± 1.16 | 83.5 ± 2.8 | 38.4 ± 1.5 |

Values are mean ± SE ($n$ = 3 plots per line under normal growth or drought stress conditions in the field). Data on grain yield per plant and spikelet fertility of homozygous $T_3$ lines and WT were analyzed by one-way ANOVA test, and the difference between each transgenic line and WT was examined by the LSD test

*, ** Significant differences between the means of lines and WT at the levels of $\alpha = 0.05$ and $\alpha = 0.01$, respectively

## Discussion

### Improved drought resistance of *OsLEA3-1*-overexpressed transgenic rice in the field

To date, there have been many reports of the development of transgenic plants with improved drought resistance by manipulation of the expression of stress related genes in laboratory or greenhouse conditions (Holmström et al. 1996; Xu et al. 1996; Shen et al. 1997; Garg et al. 2002; Dubouzet et al. 2003; Xiong and Yang 2003; Park et al. 2005). However, there are very few studies in which drought resistance of transgenic plants has been tested in the field (Wang et al. 2005; Hu et al. 2006). The results obtained under the laboratory or greenhouse conditions may be partially consistent with those obtained in the field, but must be further confirmed in drought-stressed field environments (Shao et al. 2005).

In this study, transgenic $T_1$ families over-expressing *OsLEA3-1* gene were pre-screened for drought resistance in terms of relative yield in the field, then nine homozygous $T_2$ and $T_3$ lines were tested for drought resistance in the consecutive 2 years ($T_2$ in 2005 and $T_3$ in 2006) in the field. Among the nine lines, three lines (S-18, H-8, H-24) showing significantly improved drought resistance in terms of yield in the $T_2$ generation continued to display drought resistance in the $T_3$ generation. Three lines (S-6, H-14, H-23) showing higher (but not significant) yield than WT under drought stress conditions in $T_2$ generation displayed significant drought resistance in the $T_3$ generation. The remaining three lines (S-21, A-2, A-29) did not show improved drought resistance in both years (Tables 2, 3). Some lines such as H-8 exhibited even better drought resistance in the $T_3$ generation (significance level $P < 0.01$) than in the $T_2$ generation (significance level $P < 0.05$). These results suggested that drought resistance of transgenic lines over-expressing the *OsLEA3-1* gene can be improved through selections in terms of the relative yield in the $T_1$ generation or yield in the $T_2$ or later generations under the field conditions.

Data from a number of previous studies suggested accumulation of LEA proteins was correlated with stress tolerance in oat (Maqbool et al. 2002), rice (Moons et al. 1995, 1997; Xu et al. 1996), wheat (Ried and Walker-Simmons 1993), and tobacco (Kim et al. 2005). Delayed leaf symptoms (such as wilting and dying of old leaves) caused by water-deficit stress were observed in the juvenile $R_1$ transgenic rice over-expressing a barley group 3 *LEA* gene (*HVA1*) (Xu et al. 1996). Here we also observed delayed leaf wilting in the transgenic rice lines over-expressing *OsLEA3-1* gene at the flowering stage (Fig. 4c–d). The delay of leaf wilting may allow more spikelets of the transgenic plants to develop and flower normally. Our data indeed indicated that transgenic lines had significantly higher spikelet fertility than WT.

It is interesting to note that the *OsLEA3-1* gene is located within the interval (between RM421 and RM274 on chromosome 5) of a drought tolerance-related QTL *qRSF5* for relative spikelet fertility in the Zhenshan 97/IRAT109 population (Yue et al. 2006). We are generating a near-isogenic line (IRAT109 allele in Zhenshan 97's background) to verify the function of this gene and its relationship with the QTL.

### Effect of *OsLEA3-1* controlled by different promoters in improving drought resistance at the reproductive stage of rice

In this study, three different promoters (*CaMV* 35S, *Actin1*, *HVA1-like*) were used to drive the expression of *OsLEA3-1* gene in transgenic rice for enhancing drought resistance. Grain yield and spikelet fertility were used to evaluate drought resistance under field conditions. $T_2$ and $T_3$ homozygoues lines over-expressing *OsLEA3-1* under the control of the *CaMV* 35S or the drought-inducible *HVA1-like* promoter produced significantly higher yield than WT under drought stress conditions. Generally, promoters from drought-inducible genes such as the *OsLEA3-1* in this study, *rd29A* (Yamaguchi-Shinozaki and Shinozaki 1994), *cor15a* (Baker et al. 1994), *kin1* and *cor6.6* (Wang and Cutler 1995; Wang et al. 1995) have advantages in maximizing the effects of transgenes on stress resistance improvement compared to constitutive promoters such as rice *Actin1* and maize *Ubiquitin* (Yamaguchi-Shinozaki and Shinozaki 1994; Su et al. 1998; Zhao et al. 2000a; Garg et al. 2002). Transgenes controlled by drought-inducible promoters may have strong expression only under drought stress conditions and low level of expression under normal irrigation conditions, thus minimizing possible side effects resulting from the over-expression of the target gene (Zhao et al. 2000a). We indeed observed a much higher frequency of abnormal $T_0$ plants (such as dwarfism or sterility) with the constitutive OsLEA3-A and OsLEA3-S constructs than that with the inducible OsLEA3-H construct (data not shown). In this study, the yield of some selected drought-resistance-improved lines with the OsLEA3-S construct was significant higher than that of WT under drought stress conditions (Tables 2, 3), suggesting that the OsLEA3-S construct with constitutive promoter *CaMV* 35S can also be used to generate transgenic lines with improved drought resistance.

Previously, Xu et al. (1996) reported that the over-expression of the barley *LEA* gene *HVA1* driven by the *Actin1* promoter conferred drought resistance to rice seedlings. However, in our study, the two homozygous lines over-expressing *OsLEA3-1* gene by *Actin1* promoter had no significant effect on improving drought resistance, though the yield of transgenic lines under drought stress were slightly higher than that of WT under drought stress conditions (Table 3). There are three possible explanations for this discrepancy. First, different traits measured in different environments were used to evaluate the drought resistance for the two genes. We used yield or relative yield under drought-stressed field conditions as criteria in order to evaluate the potential usefulness of the *OsLEA3-1* gene for drought resistance breeding, while the drought resistance effect measured by Xu et al. (1996) was based on growth rate and leaf damage symptoms under the water-deficit conditions in greenhouse. We also observed that some of OsLEA3-A over-expressors exhibited delayed leaf rolling during the process of drought stress development in the field (data not shown). Second, the number of $T_1$ families (16) over-expressing *OsLEA3-1* gene by *Actin1* promoter may be limited for pre-screening, considering the fact that not all the lines exhibiting over-expression of the transgene showed significantly improved drought resistance in terms of yield (e.g., OsLEA3-S line S-21). Therefore, it may be possible to identify drought-resistant lines by screening more $T_1$ families. Third, the strong activity of *Actin1* promoter (McElroy et al. 1990) may interfere with the endogenous gene expression or translation of the gene (Zhao et al. 2000a) in the two lines.

### Yield penalty associated with *Agrobacterium*-mediated transformation in rice

Although the $T_1$ families selected for drought resistance testing exhibited the same phenotypes as WT for the majority of morphological traits (e.g., plant height, plant structure, number of tillers, and flowering time), most of these transgenic families had significantly lower grain yield than WT under normal irrigation conditions (Table 1). This yield penalty may be due to several effects associated with the *Agrobacterium*-mediated transformation of rice. First, the transgenic plants were derived from tissue culture, which may have potential detrimental effects, particularly in the $T_0$ generation, on the growth and productivity (Stam et al. 1997; Liu et al. 1998; Kasuga et al. 1999; Chen et al. 2005). Second, since *Agrobacterium*-mediated transformation generates random insertions of T-DAN into the recipient genome (Hiei et al. 1994; Wu et al. 2003) and yield is associated with many genes (Yoon et al. 2006), it is possible that genes related to yield were disrupted. Third, introduction of the transgene may lead to genetic or physiological incompatibility (Holmström et al. 1996; Romero et al. 1997; Meyer 2000; Stempak et al. 2005). While some morphological changes (e.g., plant height, leaf color, erectness of stem) can be easily observed during vegetative development, changes in yield and yield-related traits may be difficult to observe.

In this study, $T_1$ families used for pre-screening exhibited normal development during vegetative growth, but the yield of most families was significantly reduced compared to WT under the non-stress conditions. Thus, selection of transgenic families for drought resistance testing should be based on not only morphological characters but also yield or yield-related traits, especially for major crops (e.g., rice, wheat, maize) in which the yield is the ultimate goal for crop production, and yield performance in drought stressed field is the most important criterion for assessing drought resistance (Turner 1979, 1997). Promising transgenic lines for drought resistance breeding should meet the following

criteria: significantly improved drought resistance, no phenotypic changes, no yield penalty, and over-expression of a single copy transgene. Having a single copy of the transgene is very important for transgenic breeding because multiple gene copies can lead to instability of expression and inheritance of transgene or even gene silencing (Stam et al. 1997; Chen et al. 2005).

Although only 30 independent $T_1$ families for each construct (about half of them have single copy of the transgene) were pre-screened in the both stressed and non-stressed fields, a few promising families meeting the above criteria were identified. If more $T_1$ families over-expressing a single copy of the transgene are included in the pre-screening, it may be possible to obtain transgenic lines with better drought resistance than the lines described here. Using this protocol, a limited number of homozygous $T_2$ and $T_3$ lines with improved drought resistance (in terms of yield and spikelet fertility) and no yield penalty were identified for formal field trail. We believe that such a bipartite (stress and non-stress) in-field screening protocol can be successfully applied to the field-testing of other transgenic crops with minor modification.

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