Heat shock proteins play an important role in plant stress tolerance and are mainly regulated by heat shock transcription factors (Hsfs). In this study, we generated transgenic rice over-expressing OsHsfA7 and carried out morphological observation and stress tolerance assays. Transgenic plants exhibited less, shorter lateral roots and root hair. Under salt treatment, over-expressing OsHsfA7 rice showed alleviative appearance of damage symptoms and higher survival rate, leaf electrical conductivity and malondialdehyde content of transgenic plants were lower than those of wild type plants. Meanwhile, transgenic rice seedlings restored normal growth but wild type plants could not be rescued after drought and re-watering treatment. These findings indicate that over-expression of OsHsfA7 gene can increase tolerance to salt and drought stresses in rice seedlings. [BMB Reports 2013; 46(1): 31-36]

INTRODUCTION
As sessile organism, plants have evolved a variety of mechanisms to rapidly respond to extreme environmental conditions by synthesizing increased amounts or new isoforms of diverse functional proteins. Among these proteins, heat shock proteins (Hsps, also known as heat stress proteins) are a group of proteins that function as molecular chaperones in regulating cellular homeostasis and promoting survival under stressful conditions (1, 2). The induction of Hsps under stresses is primarily regulated by heat shock transcription factors (Hsfs) that act by binding to the highly conserved heat shock element in the promoters of target genes (3). In many species, Hsfs are the terminal components of signal transduction chain mediating the activation of genes responsive to heat stress and a large number of chemical stressors (4-6).

Hsfs display a modular structure with an N-terminal DNA-binding domain characterized by a helix-turn-helix motif, an adjacent bipartite oligomerization domain composed of hydrophobic heptad repeats, a cluster of basic amino acid residues essential for nuclear import (the nuclear localization signal, or NLS) and a C-terminal activation domain (AHA motifs) (7, 8). In plants, the Hsf system is more complex than in any other organisms investigated so far. Based on sequence homology and domain architecture, plant Hsfs have been divided into three conserved classes, A, B and C (8). The rice genome contains thirteen class A, eight class B and four class C Hsfs. Furthermore, AHA motifs are crucial for the activity of class A Hsfs which have been reported to play a central role in the induction of various genes involved in defense under stressful conditions (9).

Many researches suggested that HsfAs not only responded to high temperature but also to oxidative stress, high salinity, chilling and other stresses. Over-expression of HsfA1 genes enhanced thermotolerance in Arabidopsis, soybean and tomato (10-12). The transgenic Arabidopsis over-expressing OsHsfA2e exhibited tolerance to high salinity stress (13). Over-expression of AtHsfA1b improved resistance to chilling in transgenic tomato (14). It was reported that over-expression of HsfA2 conferred increased tolerance to high light, salt, oxidative, osmotic and anoxia stresses (15-18). Shim et al. (19) demonstrated that transgenic rice and wheat over-expressing HsfA4a enhanced Cd tolerance.

Rice is one of the most important crops in the world. The growth and productivity of rice are often threatened by environmental factors. In the last decade, many efforts have been undertaken to generate stress tolerant rice by manipulating the expression of stress-responsive genes (13, 14, 20-22). We previously demonstrated that the expression of rice OsHsfA7 gene can be induced by heat shock treatment and other abiotic stresses (23). In this study, we generated 35S:OsHsfA7 transgenic rice plants which exhibited different phenotype and higher survival rate under abiotic stresses compared with WT. Our results suggest that transgenic rice over-expressing HsfA7 can increase resistance to high salinity and drought stresses.

RESULTS
Bioinformatics analysis of OsHsfA7
The cDNA of OsHsfA7 with accession number AK064271 is
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composed of 1,209 bp, coding for a protein of 402 aa with a predicted molecular mass of 43.9 kDa (pI 7.05). Putative HsfA7 proteins were retrieved through a BLASTP search using rice OsHsfA7 (LOC_Os01g39020) as the query. Their putative protein sequences were aligned using the DNAMAN program. OsHsfA7 shares 31.7% and 62.3% identity at the amino acid level with HsfA7 of Medicago and Sorghum. Phylogenetic analysis revealed that genetic relationship between Oryza sativa and Sorghum was closer (Fig. S1).

Root morphology in OsHsfA7-OE transgenic rice
Eight independent T0 rice transgenic lines were obtained after selection on hygromycin media and analyzed by RT-PCR. All the subsequent T1 and T2 lines showed conformed over-expression and similar root morphology and stress responses. The two highly over-expressed T2 lines (OE-1 and OE-8) were used as representative for later characterization. Root morphology of the WT and OsHsfA7-OE transgenic plant seedlings was shown in Fig. 1. OsHsfA7-OE plants exhibited longer young roots (including primary root and adventitious root) (Fig. 1A) but shorter and less lateral roots (Fig. 1B) and root hair (Fig. 1C) compared with the WT. Average radicle length of 5 d OsHsfA7-OE seedling was 3.7 cm, and that of WT was only 1.5 cm (Fig. S2A), the results showed that the young roots of transgenic seedlings grew faster than the control. Moreover, the roots of OsHsfA7-OE at tillering stage were thicker, sparser, more wide distributed and almost no lateral roots compared with that of wild type.

Enhanced salt and drought tolerance by over-expression of OsHsfA7
After irrigating with 200 mM NaCl for 10 d, leaf apex of WT plants became brown and dried, whereas over-expressing OsHsfA7 leaves remained green (Fig. 2B). Meanwhile, leaf REC and MDA content were lower in the transgenic lines than in the WT (Fig. 2E, F). In addition, hydroponic seedlings were treated with 200 mM NaCl for 24 h then transferred to 1/2 MS solution for recovery. After 4 d, leaves of WT were completely curled and wilted, while those of OsHsfA7-OE were rolled only in the tip part (Fig. 2C). After 10 d recovery, leaves of WT were withered and almost all plants were completely dead, while most of the transgenic plants remained alive and only the leaf tips scorched (Fig. 2D). The survival rates of the transgenic seedlings were 67.5% for OE-1 line and 70.8% for OE-8 line (Fig. S2B). These results indicate that over-expression of OsHsfA7 can improve salt resistance of transgenic rice.

To examine the tolerance to drought stress, three-week-old plants were withheld water for 10 d and then re-watered for additional 10 d. Both WT and transgenic plants suffered severe blast after un-watering (Fig. 3B), whereas there was remarkable

Fig. 1. Root morphology of wild-type and OsHsfA7-OE transgenic plants. (A, B) Primary root, adventitious root and lateral root in wild type and T2 transformants. Plants were grown in MS medium and photographed at 5 d (A) and 15 d (B). (C) Root hair of 7 d rice seedlings was observed by stereoscope.

Fig. 2. Influence of high salt stress on rice seedlings. (A) Seedlings before treatment. Three-week-old OsHsfA7 transgenic plants and WT control plants were grown in soil in a tray. (B) Seedlings were subjected to 200 mM NaCl for 10 d. (C, D) Hydroponic seedling treatment. WT and OsHsfA7-OE grown in 1/2 MS liquid medium for 2 weeks were treated by 200 mM NaCl for 24 h, they were photographed after recovery for 4 d (C) and 10 d (D). (E, F) Assay of REC and MDA content. Three-week-old seedlings were treated with 200 mM NaCl for 10 d. The data are the means ± SD of three independent experiments. The values with significant differences according to t-tests are indicated by asterisks (*P < 0.05; **P < 0.01).
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Fig. 3. Phenotype of the OsHsfA7 over-expression transgenic plants in response to drought treatment. Three-week-old OsHsfA7 transgenic plants and WT control plants were grown in soil in a tray. (A) Seedlings before treatment. (B) Seedlings were un-watered 10 d for drought treatment. (C) Seedlings were re-watered for 10 d after the treatment.

Fig. 4. Relative expression levels of OsHsfA7 and Hsp genes in WT and transgenic rice under normal conditions analyzed by real-time qPCR. OsUBQ5 was used as an internal control. The result is the average of three independent experiments and expression levels of these genes in WT were taken as 1, the error bars indicate ± SD. OsHsp80.2, OsHsp74.8 and OsHsp50.2 belong to Hsp90 family. OsHsp71.1, OsHsp58.7 and OsHsp23.7 belong to Hsp70 family. OsHsp26.7, OsHsp24.1 and OsHsp17.0 belong to sHsp family.

Expression of putative downstream genes regulated by OsHsfA7

To investigate the putative downstream genes regulated by OsHsfA7, nine Hsp genes from three major Hsp families (Hsp90s, Hsp70s and sHsps) were selected to detect their relative expressions in WT and OsHsfA7-OE plants under normal growth conditions by RT-PCR. It was found that only OsHsp24.1 was up-regulated in the OsHsfA7-OE plants, while expressions of the other eight Hsp genes showed no obvious difference between WT and transgenic rice (Fig. 4).

DISCUSSION

Individual Hsfs have unique functions during development. For example, HsfA9 was characterized as a specialized Hsf for embryogenesis and seed maturation in sunflower and Arabidopsis (24, 25). HsfA5 transcripts are mainly found in pollen together with HsfA4 in Arabidopsis (26). A rice HsfA4d mutant (Spl7) showed spontaneous necrotic lesions in mature leaves (27). In the present study, transgenic rice plants over-expressing OsHsfA7 exhibited different root than WT. Noticeably, lateral roots and root hair of the OsHsfA7-OE rice were shorter and less than those of the WT at seedling stage. In the other hand, tap roots of OsHsfA7-OE rice were longer compared with WT. Our results indicate that OsHsfA7 has an important role in root growth and development.

There is increasing evidence that individual member of a Hsf family may play distinct role in response to various environmental stresses (9, 13, 15, 19, 28). The growth of OsHsfA7-OE seedlings in soil was unaffected under high salt stress, while the leaf apex of the control became scorched. Meanwhile, leaf REC and MDA content were lower in the transgenic lines than in the WT. Electrolyte leakage is an indirect measure of damage done to plant cell membranes, and lower REC indicates that less membrane damage occurred (29). MDA is one of the end products of lipid peroxidation damage from free radicals (30). Salt-tolerant plants have a more perfect defense mechanism to maintain low levels of MDA (31). These findings suggested that the changes of physiological index in plants are highly adapted
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Materials and Methods

Phylogenetic analysis
OsHsfA7 homolog protein sequences from various plant species were retrieved from GenBank through a BLASTP search. OsHsfA7 and these homolog Hsfs protein sequences were aligned by DNAMAN software with default gap penalties. The phylogenetic tree was constructed by MEGA4 with the neighbor-joining algorithm using default settings. A bootstrap analysis of 1,000 replicates was performed.

Plasmid construction and rice transformation
The complete ORF of OsHsfA7 was obtained from full-length cDNA clone AK064271 (National Institute of Agrobiological Sciences, Tsukuba, Japan) by Kpn I and Pst I digestion. Then the ORF fragment was cloned into Kpn I and Pst I digested pCAMBIA1301-Multi (modified from pCAMBIA1301) under the control of the CaMV 35S promoter. The construct was transformed into rice (Oryza sativa ssp. japonica var. Nipponbare) according to the rice genetic transformation method (38).

RNA isolation and real-time qPCR analysis
OsHsfA7-OE and WT were planted according to Liu et al. (23). After 3 weeks, rice leaf samples were collected for expression analysis of OsHsfA7 and 9 OsHsp genes (primers listed in Table S1). Total RNA extraction, reverse transcription into cDNA and real-time quantitative PCR was performed according to Zou et al. (39). All cDNA samples were analyzed in triplicate from three sets of independent plants. The relative changes in gene expression were quantified using the $2^{-\Delta\Delta C_{t}}$ method. The data were expressed as mean ± standard error.

Drought, salt and heat stress treatments
T2 generation seeds of OsHsfA7-OE homozygous plants were used for stress treatments. After one week germination on 1/2 MS solid medium, the seedlings were transferred to nutritious soil in plastic pots and placed in a growth chamber (14 h-light/10 h-dark cycles) at 30°C and 75% relative humidity. After two weeks, the seedlings were used for the following abiotic stress treatments. For drought tolerance treatment, seedling plants were witheld water for 10 d, and then re-watered for 10 d. For high-salt stress treatment, seedlings were irrigated with 200 mM NaCl solution for 10 d. For heat stress treatment, plants were exposed to 47°C for 90 min. In addition, salt tolerance at seedling stage was evaluated under another stress conditions. After germination on 1/2 MS solid medium for one week, hydroponic seedlings were cultured with 1/2 MS liquid medium for 1 week, and followed by 24 h treatment in 1/2 MS solution containing 200 mM NaCl, and then transferred back to 1/2 MS solution for 4 d and 10 d recovery, respectively. The seedlings were evaluated for their survival percentage based on observations that actively growing seedlings as survivors and the non-growing and wilted seedlings were as non-survivors. All above experiments were repeated three times. The phenotype of OsHsfA7 transgenic plants and the WT under different treatments was observed and photographed.

Assay for relative electrical conductivity (REC) and malondialdehyde (MDA)
Three-week-old seedlings were treated with 200 mM NaCl for 10 d and the leaf REC and MDA were assayed. The leaf REC was measured at the beginning and at the end of salt treatment as the method described by Yu et al. (40). Five seedling shoots were harvested before and after the treatment and finely ground in liquid nitrogen using a mortar and pestle previously chilled with liquid nitrogen and the frozen powder was immediately used for MDA assay. MDA content was measured for salt treatment according to Kuk et al. (41). The mean values of REC and MDA were taken from the measurements of three replicates and ‘Standard Error’ of the means was calculated. Data were analyzed by Excel using t test to assess the significance of differences among the means.

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to stress resistance. In addition, the transgenic rice plants over-expressing OsHsfA7 had increased tolerance to drought stress in our study. These results indicate that OsHsfA7 may play a role as a member of natural defense system against high salinity and drought stresses.

Over-expression of HaSfA1 and HsfA2 genes in Arabidopsis, tomato and soybean have been reported to enhance plant heat stress resistance (14-16, 32, 33). Nevertheless, no obvious heat tolerance of OsHsfA7-OE transgenic plants was observed in our experiment. A recent investigation showed that over-expression of OsHsfA7CB improved tolerance to salt in rice (34). In addition, HsfA3 was confirmed as part of drought stress signaling (35). These results indicated that a certain Hsf could be responsible for some specific kinds of abiotic stresses and different Hsfs could have diverse role in response to abiotic stresses.

Under abiotic stresses, different Hsfs can specifically bind to heat shock elements of some Hsps and subsequently activate their transcription (36). Almoguera et al. (24) found that the expression HaHsp17.6G1 and HaHsp17.7C4 were regulated by sunflower HsfA9. Busch et al. (36) demonstrated HsfA1a and (or) HsfA1b regulated the expression of Hsp70 and Hsp101 in double mutants. Over-expression of LiHsfA2 activated the downstream genes including Hsp101, Hsp70 and Hsp25.3 (37). We have checked the expression of nine OsHsp genes in OsHsfA7-OE transgenic plants and only OsHsp24.1 was highly expressed in comparison with WT. It is likely that OsHsp24.1 was potential target gene of OsHsfA7 and involved in the adaptation to high salinity or drought stress in transgenic rice.

MATERIALS AND METHODS

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