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Overexpression of putative topoisomerase 6 genes from rice confers stress tolerance in transgenic *Arabidopsis* plants

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Database
Sequence data from this article have been deposited in the GenBank/EMBL database under the accession numbers AJ549926 (OsTOP6A1), AJ605583 (OsTOP6A2), AJ550618 (OsTOP6A3), and AJ582989 (OsTOP6B). Microarray data from this article have been deposited in Gene Expression Omnibus (GEO) repository at NCBI under the series accession number GSE5465

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DNA topoisomerase 6 (TOP6) belongs to a novel family of type II DNA topoisomerases present, other than in archaebacteria, only in plants. Here we report the isolation of full-length cDNAs encoding putative TOP6 subunits A and B from rice (*Oryza sativa* ssp. *indica*), preserving all the structural domains conserved among archaebacterial TOP6 homologs and eukaryotic meiotic recombination factor SPO11. *OsTOP6A1* was predominantly expressed in prepollinated flowers. The transcript abundance of *OsTOP6A2*, *OsTOP6A3* and *OsTOP6B* was also higher in prepollinated flowers and callus. The expression of *OsTOP6A2*, *OsTOP6A3* and *OsTOP6B* was differentially regulated by the plant hormones, auxin, cytokinin, and abscisic acid. Yeast two-hybrid analysis revealed that the full-length OsTOP6B protein interacts with both OsTOP6A2 and OsTOP6A3, but not with OsTOP6A1. The nuclear localization of OsTOP6A3 and OsTOP6B was established by the transient expression of their β-glucuronidase fusion proteins in onion epidermal cells. Overexpression of *OsTOP6A3* and *OsTOP6B* in transgenic *Arabidopsis* plants conferred reduced sensitivity to the stress hormone, abscisic acid, and tolerance to high salinity and dehydration. Moreover, the stress tolerance coincided with enhanced induction of many stress-responsive genes in transgenic *Arabidopsis* plants. In addition, microarray analysis revealed that a large number of genes are expressed differentially in transgenic plants. Taken together, our results demonstrate that *TOP6* genes play a crucial role in stress adaptation of plants by altering gene expression.

DNA topoisomerases are ubiquitous enzymes that induce transient breaks in DNA allowing DNA strands or double helices to pass through each other and re-ligate the broken strand(s). They thus relieve topological constraints in chromosomal DNA generated during many fundamental biological processes such as DNA replication, transcription, recombination and other cellular transactions. They have been classified into two types, according to their ability to cleave one (type I) or both (type II) strands of a DNA double helix [1,2]. Type II topoisomerases can be divided into two subclasses: type IIA and type IIB [3,4].

DNA topoisomerase 6 (TOP6) is the only member of the type IIB subclass found in Archaea [1,3] that generates ATP-dependent double-strand breaks with two-nucleotide overhangs in A_2B_2 heterotetrameric

Abbreviations
ABA, abscisic acid; GUS, β-glucuronidase; PP, prepollinated; TOP6, DNA topoisomerase 6.

organization [5,6]. The TOP6 subunit A (TOP6A) has only the Toprim domain [4,7] homologous to type IIA topoisomerases. Outside the Toprim domain, TOP6A shares general homology with SPO11, a protein involved in inducing double-strand breaks to initiate meiotic recombination in eukaryotes [8,9]. Their existence has also been shown in plants [10–14]. In contrast with other eukaryotes, plants contain three potential homologs of archaebacterial TOP6A in their genome [10,11]. AtSPO11-1 in *Arabidopsis* has been found to have a role in meiotic recombination [15], similar to SPO11 proteins in other eukaryotes. AtSPO11-3 and AtTOP6B are involved in endoreduplication [13] and plant growth and development [14]. However, the function of AtSPO11-2 is still not known.

Even though TOP6 has been characterized biochemically in archaebacteria, its role in eukaryotes has not yet been documented, as a homolog of subunit B is missing from all eukaryotes except plants. In this study, we isolated the homologs of archaebacterial TOP6A and TOP6B from rice (*Oryza sativa indica*), the model monocot plant. The detailed tissue-specific expression and hormonal regulation of rice TOP6 genes is reported. The interaction of subunit B with two of the subunit A homologs could also be demonstrated by the yeast two-hybrid assay. In addition, we show that the overexpression of nuclear-localized OsTOP6A3 and OsTOP6B protein genes confers increased stress tolerance in transgenic *Arabidopsis* plants.

Results

cDNA cloning

The homologs of TOP6 in rice were identified by a TBLASTN search of rice genomic sequence using the TOP6A and TOP6B protein sequences of a hyperthermophilic archaebacterium, *Sulfolobus shibatae*, as query. This search resulted in the identification of three putative homologs for TOP6A and one for TOP6B protein in rice with high sequence similarity within all the conserved motifs. The corresponding full-length cDNAs were isolated by a combination of RT-PCR and RACE, using gene-specific primers. The three subunit A genes in rice were designated *OsTOP6A1*, *OsTOP6A2*, and *OsTOP6A3*. Earlier, their orthologs in *Arabidopsis* were named as *AtSPO11-1*, *AtSPO11-2*, and *AtSPO11-3*, on the basis of their homology to meiotic recombination protein, SPO11, of *Saccharomyces cerevisiae* [10,11]. The subunit B homolog was designated *OsTOP6B*. 5′-RACE and 3′-RACE for each gene amplified a single PCR product, except for 3′-RACE of *OsTOP6A3*, which gave different-size products. The largest product was sequenced; it showed the presence of more than 10 different polyadenylation signals distributed over the entire 3′-UTR of *OsTOP6A3* (Fig. 1). Comparison of genomic (obtained from the TIGR rice genomic sequence using BLAST search tools) and cDNA sequences identified the predicted exons and introns in the *OsTOP6* genes (Fig. 1). The GenBank accession number, length of the ORF, number of exons and introns, and predicted protein length for each gene are given in supplementary Table S1. The BLAST search of the TIGR database showed that all the *TOP6* genes are represented as a single copy in the rice genome. *OsTOP6A1* and *OsTOP6A3* are located on chromosome 3 at different positions, *OsTOP6A2* on chromosome 8, and *OsTOP6B* on chromosome 9 (supplementary Table S1).

Sequence analysis

The multiple sequence alignment of the deduced amino-acid sequences of the three OsTOP6A proteins showed the presence of all five conserved motifs and residues (supplementary Fig. S1), found in other SPO11/TOP6A homologs [3,4,7,16]. Overall, rice TOP6A amino-acid sequences are 56–68% identical with *Arabidopsis* SPO11 homologs, 18–32% with animal proteins, 13–24% with yeast SPO11 proteins, and 16–27% with archaebacterial TOP6A proteins.

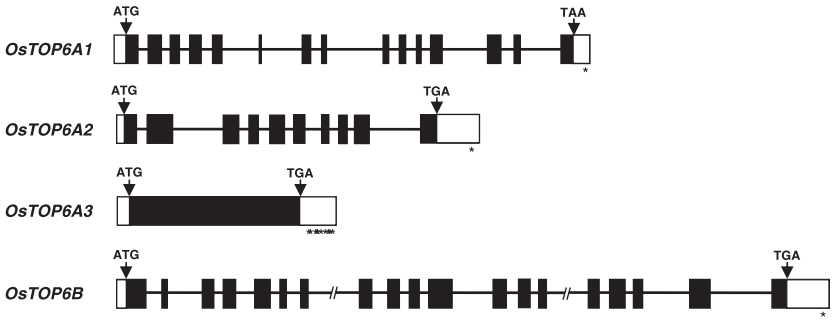


Fig. 1. The exon–intron organization of putative rice *TOP6A* and *TOP6B* genes. The coding and untranslated regions are represented by black and open boxes, respectively. The introns are represented by lines. Start and stop codons are indicated by arrows. Polyadenylation signals are represented by asterisks. The two large introns in the *OsTOP6B* gene are represented by interrupted lines.

The regional similarity was much higher particularly in the five conserved motifs. OsTOP6A proteins contain the active tyrosine residue within the CAP domain, which is invariant among other SPO11 homologs and has been shown to be necessary for double-strand break induction in *S. cerevisiae* [3,16]. The conserved DXD sequence of the Toprim domain, which is thought to co-ordinate Mg^{2+} ion required for DNA binding and may also assist in strand cleavage and re-ligation reactions [4], was present in OsTOP6A1 and OsTOP6A3, but absent from OsTOP6A2. Notably, OsTOP6A3 protein showed the presence of an N-terminal extension that is not present in OsTOP6A1 and OsTOP6A2. The OsTOP6B protein also harbors all the conserved domains (N-terminal GHKL, middle H2TH, and C-terminal transducer domain) and the motifs of the Bergerat fold (motif B1-B3) found in other TOP6B homologs (Fig. S1) [3,11], showing an overall sequence identity of 69.6% with *Arabidopsis* and 15–30% with archaebacterial TOP6B homologs.

The amino-acid sequence analysis of rice TOP6 proteins also predicted several potential putative phosphorylation sites for casein kinase II, protein kinase C, tyrosine kinase, histidine kinase, cAMP-dependent and cGMP-dependent protein kinases, and putative N-glycosylation, N-myristoylation and amidation. It is known from other studies that the activity of topoisomerases is modulated by these post-translational modifications [17,18]. These potential post-translational modification sites in the primary amino-acid sequence remain to be functionally validated.

Intron conservation and phylogenetic analysis

The position and phasing of introns was found to be highly conserved between the respective rice and *Arabidopsis SPO11/TOP6* genes (Fig. S2), suggesting that these genes may have evolved from a common ancestor. The *AtSPO11-1* and *AtSPO11-2* genes were previously found to possess one intron in their 3′-UTRs [10]. However, no intron was found in the 3′-UTRs of *OsTOP6A1* and *OsTOP6A2*, as a single 3′-RACE product was amplified for both genes in repeated experiments. Also, intron 2 of *AtSPO11-2* and the only intron present in the ORF of *AtSPO11-3* genes (Fig. S2) are absent from rice *OsTOP6A2* and *OsTOP6A3* genes, respectively. From these observations, it can be speculated that *Arabidopsis* has gained the intron present in the 3′-UTRs of *AtSPO11-1* (intron 15) and *AtSPO11-2* (intron 11), and rice has lost intron 2 and intron 1 from the *OsTOP6A2* and *OsTOP6A3* genes, respectively, during the course of evolution after divergence into dicots and monocots, according to the assumptions of Hartung *et al.* [19]. Phylogenetic analysis of SPO11/TOP6A homologs from different organisms (Fig. S3) showed that OsTOP6A1 is more closely related to SPO11 homologs from other organisms, whereas OsTOP6A2 and OsTOP6A3 were more closely related to archaebacterial TOP6A proteins. Moreover, OsTOP6A proteins are significantly more closely related to SPO11/TOP6A proteins from other organisms than each other, indicating that *TOP6A* genes in rice did not arise by recent duplications, but rather represent ancient paralogs. Also, OsTOP6B appears to be closely related to AtTOP6B and archaebacterial TOP6B proteins. Other than in plants, TOP6B protein is only present in archaebacteria. Thus, it can be speculated that TOP6 was acquired by plants from Archaea by lateral gene transfer. From a comparison of intron positions and phylogenetic trees, it has been postulated that the evolution of *AtSPO11-1* and *AtSPO11-2* (orthologs of *OsTOP6A1* and *OsTOP6A2*) in *Arabidopsis* occurred as the result of duplication of an ancestral *SPO11* gene present in the last common ancestor of plants and animals, shortly after the divergence of plants and animals [19]. The evolution of *AtSPO11-3* (ortholog of *OsTOP6A3*) has been proposed to have occurred by reintegration of a partially spliced mRNA of *AtSPO11-2* into the genome by a reverse transcription mechanism [19]. However, the evolution of *TOP6* genes in plants remains a matter of debate. Sequencing of complete genomes of other organisms, including lower plants, will hopefully help to answer this question.

Tissue-specific expression and hormonal regulation

To examine the expression of *OsTOP6* genes in different plant organs, quantitative real-time RT-PCR analysis was performed from total RNA isolated from 6-day-old seedlings, young roots, young shoots, callus, prepollinated (PP) and postfertilized flowers. This analysis showed that the *OsTOP6A1* gene was predominantly expressed in PP flowers (Fig. 2A,C), which are principally composed of meiotic cells. However, it was also found to be expressed in tissues other than PP flowers, although at lower level (Fig. 2A,C). Several larger transcripts were also found at low levels in PP flowers and other tissues examined by semi-quantitative RT-PCR using gene-specific primers (Fig. 2A). Similar observations have been made in the case of *Arabidopsis* [10] and mammalian [20] SPO11 homologs. However, the biological significance of these alternat-

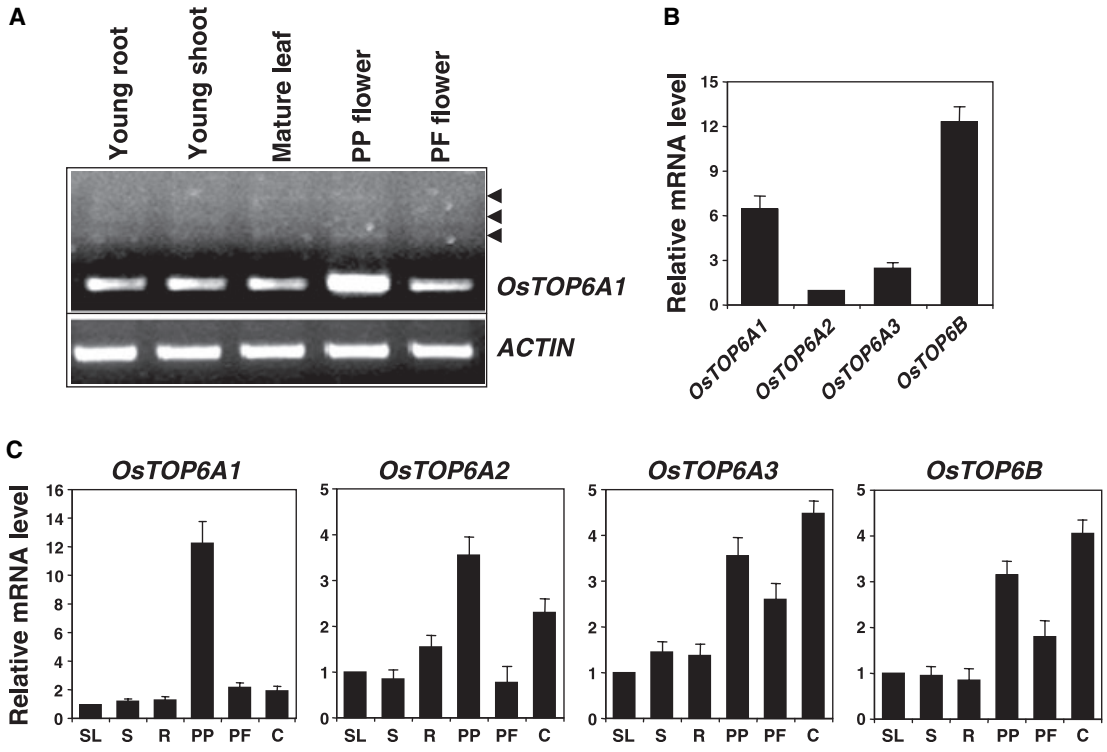


Fig. 2. Tissue-specific expression of *OsTOP6* genes. (A) Semi-quantitative RT-PCR analysis of *OsTOP6A1* in different tissues (indicated at the top of each lane) using gene-specific primers. Arrowheads represent alternative transcripts of *OsTOP6A1*. *ACTIN* represents the internal control. (B) Relative expression of the four rice *TOP6* genes in PP flowers assessed using real-time PCR. mRNA levels were calculated relative to the expression of *OsTOP6A2*. (C) Quantitative real-time RT-PCR analysis for expression of individual rice *TOP6* genes in different tissues as indicated below each bar (SL, 6-day-old seedlings; S, young shoots; R, young roots; PP, prepollinated flowers; PF, postfertilized flowers; C, callus). The mRNA levels in different tissues for each candidate gene were calculated relative to the expression in 6-day-old seedlings. For each tissue, the same cDNA sample was used to study the expression of the different genes.

ive transcripts is not known. *OsTOP6A2* is expressed at much lower level than other *OsTOP6* genes in all the tissues examined, as exemplified by comparative analysis of the expression data obtained with PP flowers (Fig. 2B). *OsTOP6A2* was found to be expressed in PP flowers and callus at significant levels (Fig. 2C). This indicates that it may have a role in meiosis and somatic cell division. *OsTOP6A3* and *OsTOP6B* were constitutively expressed in all the plant tissues/organs tested, although quantitative variation in transcript levels was observed (Fig. 2C).

Further, real-time PCR analysis was performed to quantify the mRNA concentrations of Os*TOP6* genes after treatment of rice seedlings with different plant hormones (Fig. 3). *OsTOP6A1* did not show any response to the hormones tested in this study. However, the transcript levels of *OsTOP6A2*, *OsTOP6A3* and *OsTOP6B* were up-regulated 2–3-fold after treatment with auxin and cytokinin (Fig. 3), indicating their role in cell division. Also, the transcript abundance of *OsTOP6A3* and *OsTOP6B* increased up to 3–5-fold in the presence of abscisic acid (ABA) within 3 h in rice seedlings (Fig. 3).

Interaction of rice TOP6B protein with TOP6A homologs

TOP6 in archaebacteria causes double-strand breaks in heterotetrameric (A_2B_2) form [5,6]. To ascertain the possible interaction of putative TOP6B with TOP6A homologs in rice, yeast two-hybrid analysis was performed. The results clearly show that OsTOP6B only interacts with the OsTOP6A2 and OsTOP6A3 but not with OsTOP6A1 (Fig. 4), an observation essentially similar to that reported in *Arabidopsis* [11]. However, we could not detect the interaction of partial OsTOP6B (pTOP6B, amino acids 1–420) lacking the C-terminal transducer domain with any of the OsTOP6A homologs (Fig. 4). It substantiates the idea that the transducer domain of TOP6B is involved in interaction with TOP6A and structurally transduces appropriate signals to it [21].

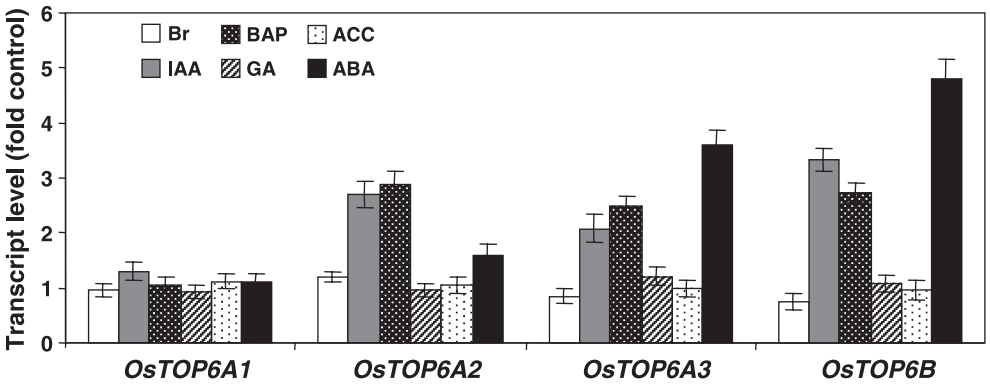
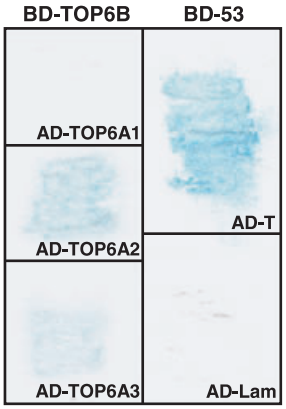


Fig. 3. Hormonal regulation of *OsTOP6* genes. Total RNA extracted from 6-day-old light-grown seedlings harvested after treatment with 10 μM epibrassinolide (Br), 50 μM indole-3-acetic acid (IAA), 50 μM benzylaminopurine (BAP), 50 μM gibberellic acid (GA), 50 μM 1-aminocyclopropane-1-carboxylic acid (ACC), or 50 μM abscisic acid (ABA) for 3 h was used for real-time PCR quantification of expression levels. mRNA levels were calculated relative to the expression in mock-treated rice seedlings (kept in water) for each gene. For each tissue, the same cDNA sample was used to study the expression of the different genes.



DNA binding domain	Activation domain	X-Gal reaction
BD-53	AD-T	+
BD-53	AD-Lam	-
BD-TOP6B	AD-TOP6A1	-
BD-TOP6B	AD-TOP6A2	+
BD-TOP6B	AD-TOP6A3	+
BD-pTOP6B	AD-TOP6A1	-
BD-pTOP6B	AD-TOP6A2	-
BD-pTOP6B	AD-TOP6A3	-

Fig. 4. Yeast two-hybrid analysis showing the interaction of OsTOP6B protein with OsTOP6A2 and OsTOP6A3. AD-TOP6A1, AD-TOP6A2 and AD-TOP6A3 denote the fusion of full-length OsTOP6A1, OsTOP6A2 and OsTOP6A3 with GAL4 activation domain, respectively. BD-TOP6B and BD-pTOP6B represents the fusion of full-length and partial OsTOP6B with GAL4 DNA-binding domain, respectively. The interaction of BD-53 (fusion of p53 with GAL4 DNA-binding domain) with AD-T (fusion of antigen T with activation domain) and AD-Lam (fusion of lamin C with activation domain) represents the +ve and –ve controls, respectively.

Subcellular localization of OsTOP6A3 and OsTOP6B proteins

The *OsTOP6A3* and *OsTOP6B* genes encode highly basic (OsTOP6A3, pI 9.30; OsTOP6B, 8.94) proteins. To establish the subcellular localization of these proteins, the complete ORFs of these genes were fused in-frame with the β-glucuronidase (*GUS*) gene, and expressed transiently under the control of CaMV 35S promoter. The recombinant vectors and pCAMBIA 3301 (cytosolic control) were bombarded into the inner epidermal cells of white onion. Subcellular localization of fusion proteins (OsTOP6A3::GUS and OsTOP6B::GUS) and GUS protein was established using GUS histochemical assay buffer. Both the fusion proteins were found to be concentrated in the nucleus, whereas the GUS protein alone was distributed all over the cell (Fig. 5). Staining with the nucleus-specific dye Hoechst 33258 confirmed the nuclear localization.

Overexpression of *OsTOP6A3* and *OsTOP6B* in *Arabidopsis*

To establish the functional significance of the TOP6A and TOP6B homologs, OsTOP6A3 and OsTOP6B, respectively, we generated transgenic *Arabidopsis* plants in which the complete ORFs of *OsTOP6A3* and *OsTOP6B* were overexpressed under the control of

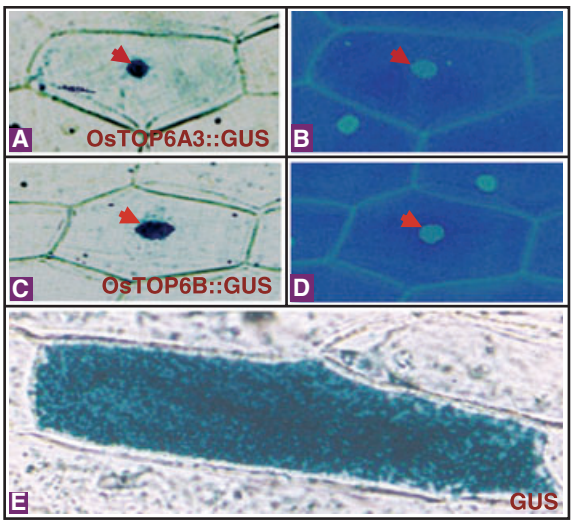


Fig. 5. Subcellular localization of OsTOP6A3 and OsTOP6B proteins. (A) and (C) represent the localization of OsTOP6A3::GUS and OsTOP6B::GUS fusion proteins, respectively. (E) Localization of GUS protein. (B) and (D) show Hoechst 33258 staining of (A) and (C), respectively.

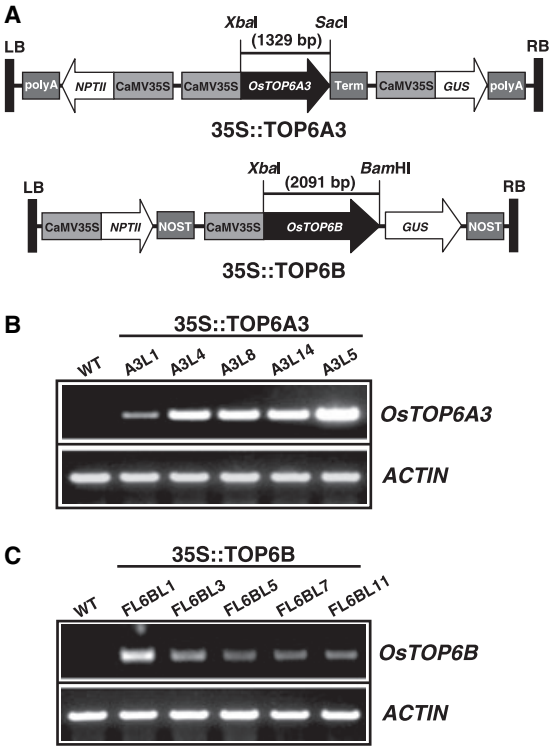


Fig. 6. Overexpression of *OsTOP6A3* and *OsTOP6B* cDNAs in transgenic *Arabidopsis* plants. (A) Schematic representation of the constructs used to overexpress *OsTOP6A3* (35S::TOP6A3) and *OsTOP6B* (35S::TOP6B) in *Arabidopsis*. (B) and (C) Semi-quantitative RT-PCR analysis showing the expression of *OsTOP6A3* and *OsTOP6B* in wild-type and five randomly selected transgenic lines using gene-specific primers. *ACTIN* represents the internal control.

CaMV 35S promoter (35S::TOP6A3 and 35S::TOP6B) by the floral-dip transformation method (Fig. 6A). A total of 22 and 24 independently transformed kanamycin-resistant T1 transgenic plants were obtained for 35S::TOP6A3 and 35S::TOP6B, respectively. The presence of transgene in kanamycin-resistant *Arabidopsis* plants was confirmed by PCR (data not shown). All the T1 transgenic plants of the same construct exhibited similar morphological and growth characteristics. Therefore, from these, only five plants were selected randomly for each (35S::TOP6A3 and 35S::TOP6B) and allowed to grow to obtain homozygous lines for subsequent analysis. Semi-quantitative RT-PCR analysis confirmed the overexpression of transgenes in the transgenic plants (Fig. 6B,C). The transgenic plants harboring 35S::OsTOP6A3 did not show any significant effect on growth compared with wild-type plants. However, 35S::TOP6B transgenic plants exhibited slight growth retardation.

Abiotic stress tolerance of transgenic *Arabidopsis* plants

The effect of different abiotic stresses was assessed on homozygous 35S::TOP6A3 and 35S::TOP6B transgenic *Arabidopsis* plants. Analysis of the transgenic plants revealed that overexpression of *OsTOP6A3* and *OsTOP6B* reduced the ABA sensitivity of seed germination (Fig. 7A) and root growth (Fig. 7B). As the stress hormone, ABA, has been implicated in various plant responses to many environmental stresses, including high salinity and dehydration, we sought to determine the response of transgenic plants to other environmental stresses also.

Evaluation of the overexpression of transgenic plants for salt stress tolerance revealed that the percentage germination of the transgenic plants was much higher than the wild-type on Murashige–Skoog (MS) medium supplemented with different concentrations of NaCl (Fig. 8). The increased salt tolerance of the transgenic plants with respect to wild-type was apparent at NaCl concentrations of 150–250 mM. After 3 days, only the transgenic plants showed 16–25% germination at 250 mM NaCl (Fig. 8A). After 6 days of growth on MS medium supplemented with 150, 200 and 250 mM NaCl, the transgenic seedlings were healthier and exhibited 39–48% germination on 250 mM NaCl compared with only 9% for the wild-type (Fig. 8B).

The tolerance to dehydration stress was determined in terms of relative fresh weight of stressed transgenic

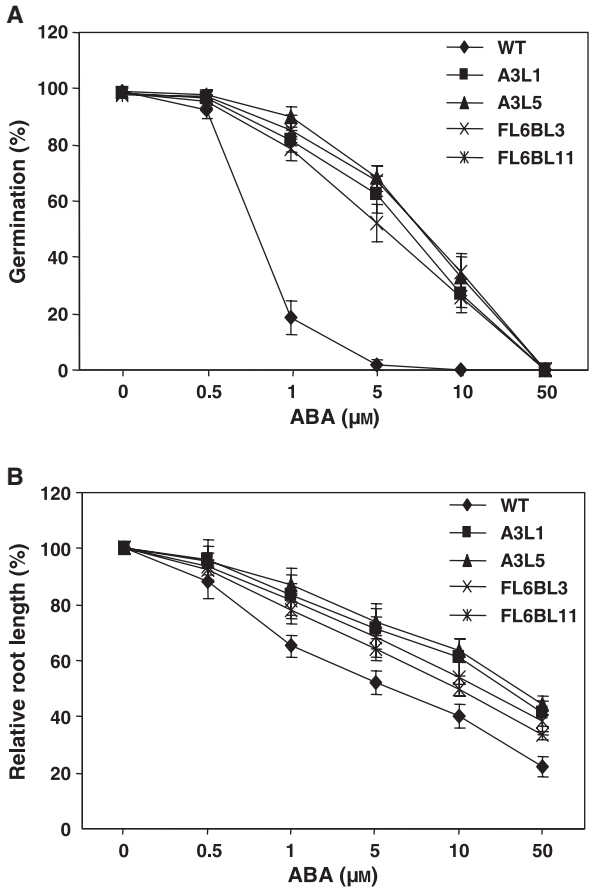


Fig. 7. Effect of ABA on wild-type and transgenic *Arabidopsis* overexpressing *OsTOP6A3 and OsTOP6B*. (A) ABA dose–response for inhibition of germination. The number of germinated seeds (with fully emerged radicle tip) was expressed as the percentage of the total number of seeds plated (40–80). (B) Inhibition of root growth. Root length of ABA-treated seedlings was expressed as a percentage of controls incubated on ABA-free medium. Values are mean ± SD for 12 seedlings each. Data from two representative transgenic lines for both 35S::TOP6A3 (A3L1 and A3L5) and 35S::TOP6B (FL6BL3 and FL6BL11) plants are presented.

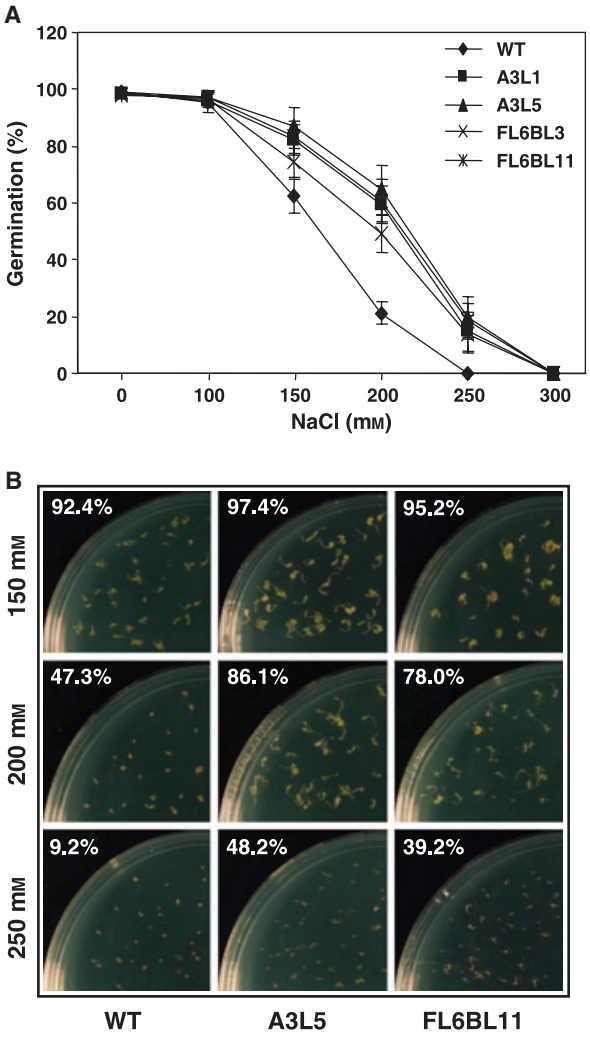


Fig. 8. Salt stress tolerance of wild-type and transgenic plants overexpressing *OsTOP6A3* and *OsTOP6B*. (A) Percentage germination of wild-type and transgenic seeds on MS medium supplemented with various concentrations of NaCl after 3 days. (B) The wild-type and transgenic plants (representative A3L5 and FL6BL11 lines) were grown on MS plates supplemented with various concentrations of NaCl (indicated on the left) for 6 days. The mean percentage germination from three independent experiments is given in the respective box.

and wild-type seedlings compared with nonstressed seedlings. The relative fresh weight of the transgenic seedlings grown on medium supplemented with 100, 200, and 300 mM mannitol was always higher than that of the wild-type seedlings (Fig. 9), which confirmed the ability of transgenic plants to tolerate dehydration stress. Although, the transgenic lines of each construct tested in this study showed different transcript levels of the transgene (Fig. 6B,C), no significant difference in their sensitivity to ABA and tolerance to salt and dehydration stress was observed (Figs 7–9); this was also valid for other transgenic lines tested for which the data have not been presented.

Expression of stress-responsive genes in transgenic plants

The induction of numerous stress-responsive genes is a hallmark of stress adaptation in plants. To elucidate further the role of *OsTOP6A3* and *OsTOP6B* in stress tolerance, we examined the transcript levels of some *Arabidopsis* stress-inducible genes, namely *COR15A*, *DREB1A*, *RD29A*, *KIN1*, *KIN2*, and *ERD10*, in wild-type and transgenic plants. Although the transcript

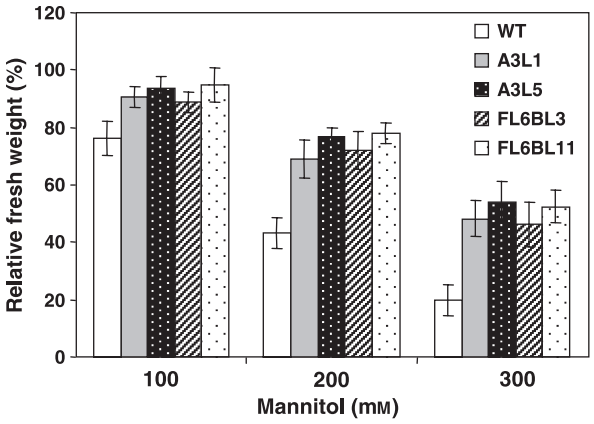


Fig. 9. Dehydration stress tolerance of wild-type and transgenic plants overexpressing *OsTOP6A3* and *OsTOP6B*. Percentage fresh weight of 8-day-old seedlings germinated on different concentrations of mannitol relative to the fresh weight of unstressed seedlings grown on MS is given. Values are mean ± SD for 12 seedlings each.

levels of these genes in transgenic plants did not show any significant change compared with wild-type under normal growth conditions, the expression of all these genes increased to a much higher degree in transgenic plants than in wild-type under different stress conditions (Fig. 10). The stress tolerance of the overexpressing plants may be enhanced, at least in part, by the high-level accumulation of these gene products in response to stress.

Microarray analysis

The effect of overexpression of *OsTOP6A3* and *OsTOP6B* cDNAs under normal growth conditions was analyzed on the transcription of 22 500 genes of *Arabidopsis* by microarray analysis performed with the total RNA isolated from the transgenic and wild-type plants. The data analysis revealed that a total of 240 and 229 genes exhibit a significant change in expression (more than twofold, $P < 0.01$) between wild-type and 35S::TOP6A3 and 35S::TOP6B transgenic plants, respectively (Fig. 11A, supplementary Table S2). These gene products include proteins involved in abiotic or biotic stress response, protein metabolism, transport, transcriptional regulation, signal transduction, cell organization and biogenesis, and other physiological or metabolic processes (supplementary Table S2). We also found many genes with unknown functions to be differentially expressed in transgenic plants. Further analysis revealed that 147 genes showing differential expression (91 up-regulated and 56 down-regulated)

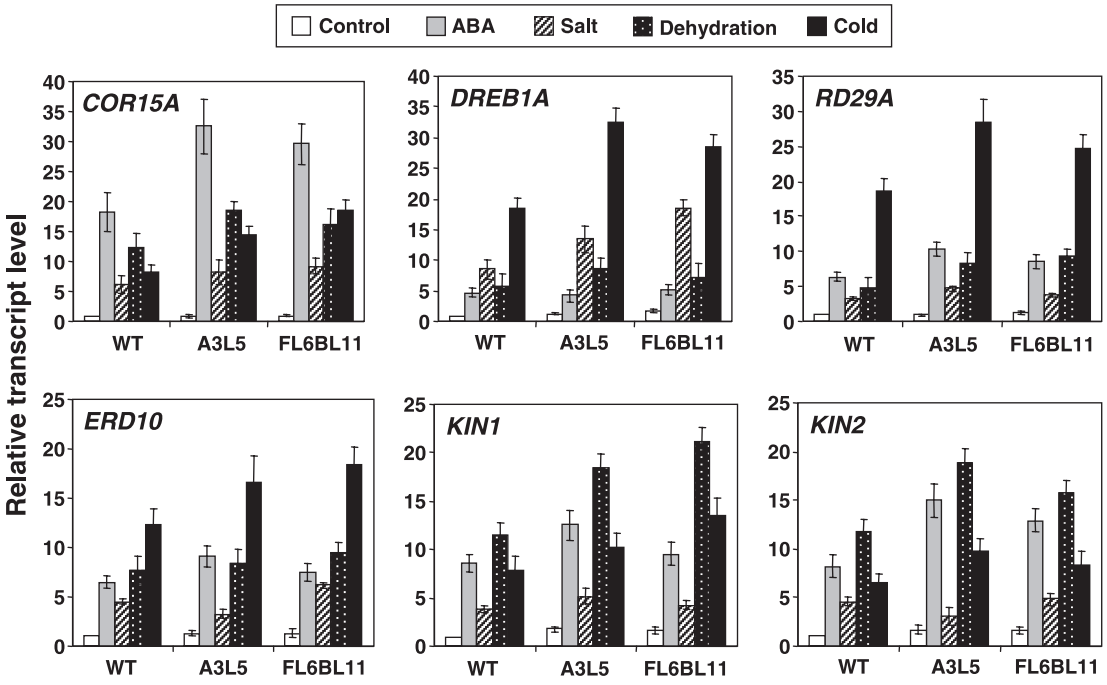


Fig. 10. Expression profiles of stress-responsive genes in wild-type and transgenic plants. Control, untreated; ABA, 100 μM ABA for 2 h; Salt, 200 mM NaCl for 2 h; Dehydration, 300 mM mannitol for 2 h; Cold, 4 °C for 4 h. Real-time PCR analysis was performed using gene-specific primers. The mRNA levels for each gene in transgenic (A3L5 and FL6BL11) plants were calculated relative to the expression in control wild-type plants. The same cDNA sample was used to study the expression of different genes for each RNA sample.

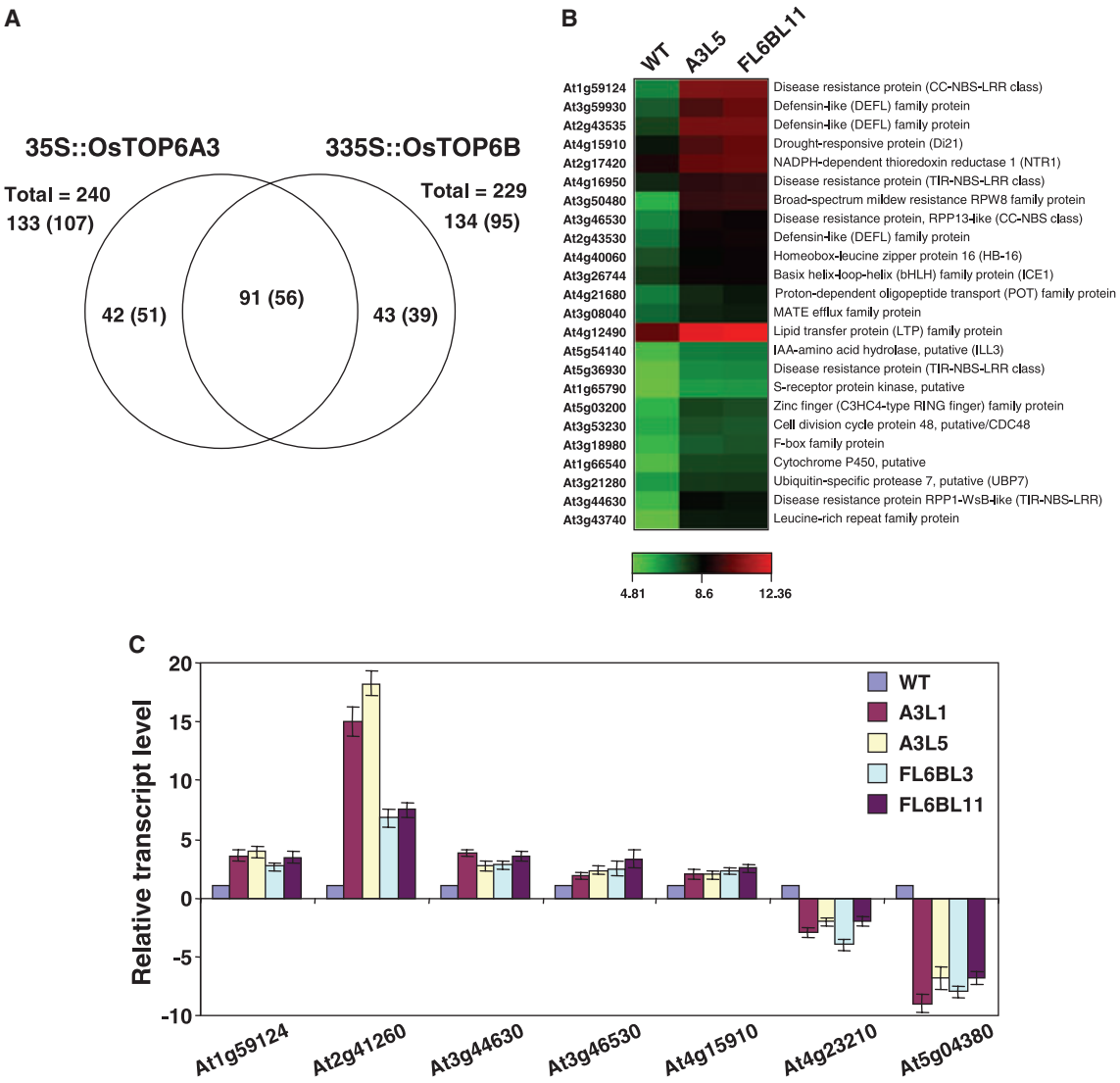


Fig. 11. (A) Venn diagram showing the number of differentially expressed genes (more than two fold with $P < 0.01$) in transgenic plants. Numbers outside and inside the parentheses indicate number of up-regulated and down-regulated genes, respectively. (B) Overview of the stress-related genes showing differential expression in both transgenic plants (A3L5 and FL6BL11) by cluster display. (C) Real-time PCR analysis of expression profiles of selected genes from microarray analysis in wild-type and transgenic plants. The mRNA levels for each gene in the transgenic plants were calculated relative to the expression in the wild-type plants. The same cDNA sample was used to study the expression of different genes for each RNA sample.

were common for 35S::TOP6A3 and 35S::TOP6B transgenic plants as shown in a Venn diagram (Fig. 11A, supplementary Table S2). The genes differentially expressed in both the transgenic plants represent different functional categories, with stress-related genes being more predominant (supplementary Table S2). The expression profile of some of the stress-related genes up-regulated in both transgenic plants are shown in Fig. 11B. The expression of *COR15A*, *DREB1A*, *RD29A*, *KIN1*, *KIN2*, and *ERD10* was not found to be altered in microarray analysis, as also observed by real-time PCR (Fig. 10). The real-time PCR analysis was performed to confirm the results obtained by microarray analysis by analyzing the expression of some genes identified by microarray analysis, in the wild-type and transgenic plants. Essentially the same expression patterns of all the genes analyzed were observed in the two independent lines each for 35S::TOP6A3 and 35S::TOP6B transgenic plants, as that obtained from microarray analysis (Fig. 11C).

Discussion

Although TOP6 activity is well characterized in archaebacteria, its existence in eukaryotes is still debatable, because the homolog of subunit B is absent from all eukaryotes except plants. The absence of TOP6 from eukaryotes other than plants shows that either this enzyme complex is not required or other factors have assumed its function. In this study, we have identified and characterized three putative TOP6A homologs (OsTOP6A1, OsTOP6A2, and OsTOP6A3) and one TOP6B homolog (OsTOP6B) in rice that contain all the conserved motifs and residues. Phylogenetic analysis revealed that OsTOP6A1 in rice and AtSPO11-1 in *Arabidopsis* represent the functional homolog of SPO11 protein present in other organisms. Real-time PCR analysis showed that *OsTOP6A1* is expressed predominantly in PP flowers which are composed of meiotic cells. This is consistent with earlier observations on the role of SPO11 protein in meiotic recombination in *Arabidopsis* and other eukaryotes [8,9,15]. Grelon *et al*. [15] showed that in the *Arabidopsis spo11–1* null mutant, some bivalents are also formed. In contrast, no meiotic recombination event takes place in *spo11* mutants of yeast, *Drosophila* and *Caenorhabditis elegans* [22,23], as only one *SPO11* gene is present in other eukaryotes. Although the expression of *OsTOP6A2* in PP flowers supported the idea that it may act redundantly to OsTOP6A1 for meiotic recombination, its exact role remains to be demonstrated. The constitutive expression of *OsTOP6A3* and *OsTOP6B* at higher levels in all plant tissues/organs indicates their role in cell proliferation and overall growth and development in plants. Their orthologs in *Arabidopsis* have a crucial role in brassinosteroid-mediated growth and development [14]. The transcript levels of *OsTOP6A2*, *OsTOP6A3*, and *OsTOP6B* increased in response to auxin and cytokinin, indicating their role in cell proliferation and hormone signaling. The interaction of OsTOP6A3 with OsTOP6B along with their similar expression patterns and localization in the nucleus suggest that they may represent the functional homologs of archaebacterial TOP6 in rice, involved in topological manipulation of DNA. This idea is supported by similar predicted functions of AtSPO11-3 and AtTOP6B in *Arabidopsis* by analysis of mutants of these genes [12–14].

To study the function of putative TOP6A and TOP6B homologs, *OsTOP6A3* and *OsTOP6B* cDNAs were overexpressed in *Arabidopsis*. The transgenic *Arabidopsis* plants overexpressing *OsTOP6A3* and *OsTOP6B* exhibited reduced sensitivity to the stress hormone, ABA, as indicated by the higher percentage seed germination and root growth in the presence of ABA. Also, the transgenic plants performed better than the wild-type under various stress conditions. The increased salinity tolerance was evident from the higher percentage of seed germination and green and healthier seedlings on MS medium supplemented with NaCl. The fresh weight of transgenic seedlings was always higher than the wild-type when subjected to dehydration stress. In addition, expression of many stress-responsive genes was found to be more rapidly induced under stress conditions in transgenic plants. Microarray analysis revealed that overexpression of *OsTOP6A3* and *OsTOP6B* alters the expression of a large number of *Arabidopsis* genes including many abiotic and biotic stress-related genes.

The development and survival of plants is constantly challenged by changes in environmental conditions. To respond and adapt or tolerate adverse environmental conditions, plants elicit various physiological, biochemical and molecular responses, leading to changes in gene expression. The products of a number of stress-inducible genes counteract environmental stresses by regulating gene expression and signal transduction in the stress response. Because abiotic stresses affect cellular gene expression machinery, it is evident that genes involved in nucleic acid processing such as replication, repair, recombination, and transcription are likely to be affected as well. Several nucleic acid processing enzymes such as RNA and DNA helicases from various organisms have been shown to respond to different abiotic stresses [24–28]. Recently, the promoter of pea topoisomerase II has been shown to respond to various abiotic stresses [29]. Most of the stress-related genes are rapidly induced within a short period of exposure to stress [30–34]. However, the expression of *OsTOP6* genes in rice seedlings is not altered on exposure to different stresses (data not shown), except for induction by ABA, under our experimental conditions. Expression of *Arabidopsis HOS9* (homeodomain transcription factor gene) and *HOS10* (R2R3-type MYB transcription factor gene) was also not found to be affected by different stress treatments in wild-type plants, although they mediate stress tolerance in *Arabidopsis* [35,36].

It has been well demonstrated that both subunits A and B are required for TOP6 activity in archaebacteria [5,6]. Although TOP6 activity has not been demonstrated in plants, both subunits are required for regulation of plant growth and development and endoreduplication in *Arabidopsis* [12–14]. Recently, another protein, RHL1 (root hairless 1), has been found to be an essential component of the plant DNA TOP6 complex [37]. However, our study shows that the overexpression of only one or the other subunit of rice TOP6 can impart stress

tolerance to transgenic *Arabidopsis* plants, independently of each other. This can be explained in one of two ways: (a) these proteins can regulate cellular processes independently or may associate with protein complexes other than TOP6 to alter gene expression; or (b) there must be a minimum threshold of TOP6 subunit A or B proteins above the wild-type levels, such as are likely to be present in 35S::TOP6A3 and 35S::TOP6B transgenic plants, to confer stress tolerance.

Although the exact mechanism of stress tolerance mediated by OsTOP6A3 and OsTOP6B is not understood, several possible explanations can be given. The most likely is that, being the homologs of TOP6, the overexpression of these proteins may cause chromatin modification by introducing double-strand DNA breaks directly or in association with other proteins in the nucleus, influencing the expression level of several genes under normal and stress conditions. This explanation is supported by the demonstration of the altered expression of a large number of genes by overexpression of *OsTOP6A3* and *OsTOP6B* genes (present study) and the mutation in *AtSPO11-3* and *AtTOP6B* [14] in *Arabidopsis*. The improved stress tolerance of transgenic *Arabidopsis* plants may partly be explained by enhanced induction of stress-inducible genes, such as *COR15A*, *DREB1A*, *RD29A*, *ERD10*, *KIN1*, and *KIN2*, analyzed in this study, under stress conditions. The differentially expressed genes in transgenic plants that encode proteins that probably function in stress tolerance include late embryogenesis-abundant (LEA) proteins, defensins, transporters, senescence-related genes, protease inhibitors, lipid-transfer proteins, transcription factors, and several disease-resistance proteins. These proteins have been shown to be involved in eliciting several physiological, biochemical and molecular changes at the cellular level, including protection of macromolecules such as enzymes and lipids, maintenance of osmotic pressure, protein turnover and recycling of amino acids, and inhibition of proteases under stress conditions [30–34,38]. Further, various transcription factors up-regulated in transgenic plants are involved in regulation of signal transduction and gene expression that can modulate stress responses. For example, the transcription factor ICE1 [inducer of C-repeat binding factor (CBF) expression 1], which acts upstream of CBFs in the cold-response pathway and regulates transcription of a large number of genes [39,40], is up-regulated in transgenic plants that overexpress OsTOP6A3 and OsTOP6B and may provide stress tolerance.

The other possible explanation is based on the observations that the overexpression of *OsTOP6A3* and *OsTOP6B* cDNAs imparts reduced sensitivity to ABA in the transgenic plants and their transcript abundance is increased in response to ABA in rice seedlings. Also, the mutants, *AtSPO11-3* and *AtTOP6B*, were found to be hypersensitive to ABA in a previous study [14]. The plant hormone ABA is also known to modulate cellular gene expression under multiple stress conditions such as salinity, dehydration and cold [32,41]. Taken together, these results provide the first insight into the involvement of rice *TOP6* genes in ABA-dependent processes that provide stress tolerance to transgenic plants. The third possible explanation is based on the observation that the mutants of orthologs of rice *OsTOP6A3* and *OsTOP6B*, *AtTOP6B* and *AtSPO11-3,* were found to be partially insensitive to applied brassinosteroids [14] and may have a role in brassinosteroid signaling. Brassinosteroids act through a multicomponent signaling pathway to regulate the expression of a large set of genes involved in critical processes of plant growth and development [42]. Several studies have shown that brassinosteroids are also implicated in modulation of stress responses such as cold stress, heat stress, salt stress, oxidative stress, and pathogen infection [43–47]. Recently, it has been reported that loss-of-function mutations in the *DET2* gene, which is involved in brassinosteroid biosynthesis, provides enhanced resistance to oxidative stress in *Arabidopsis* [48]. In the light of these observations, it can be speculated that the overexpression of *OsTOP6A3* and *OsTOP6B* may modulate the brassinosteroid signal-transduction pathway which, in turn, activates the constitutive expression of some abiotic and biotic stress-related genes in transgenic *Arabidopsis* plants, providing stress tolerance. However, the exact molecular mechanisms underlying these explanations remain to be elucidated.

Abiotic stresses such as drought, high salinity and low temperature are the most common environmental stress factors limiting crop productivity throughout the world. The identification of novel genes involved in environmental stress responses provides the basis for effective engineering strategies for improving stress tolerance in crop plants [31–34,49]. The ectopic expression of several genes from different plant species, including tobacco, *Arabidopsis*, *Brassica*, pea, barley and rice, in transgenic plants has been shown to confer multiple stress tolerance [28,50–55]. The present study provides evidence that the overexpression of *OsTOP6A3* and *OsTOP6B* confers stress tolerance in transgenic *Arabidopsis* plants and may be used to engineer stress tolerance in crop plants. Furthermore, for a better understanding of the functions of *TOP6* genes, transgenic rice plants should be generated and their target genes identified.

Experimental procedures

Plant materials and growth conditions

Rice (*Oryza sativa* ssp. *indica* var. Pusa Basmati-1) seeds were treated and grown as described [56]. *Arabidopsis thaliana* (L) Heynh. ecotype Columbia (Col) was used for raising transgenic plants. *Arabidopsis* plants were grown in a culture room under constant illumination (≈ 80 μmol·m^{-2}·s^{-1}), maintained at 22 ± 1 °C, in clay pots containing Soilrite (Kelperlite, Banglore, India; 1 : 1 : 1 ratio of vermiculite, perlite and *Sphagnum* moss) supplemented with nutrient medium. To germinate seedlings on Petri plates under aseptic conditions, seeds were surface sterilized in a solution containing 2% sodium hypochlorite and 0.01% Triton X-100 for 5 min, and rinsed with sterile water at least five times. Seeds were then suspended in 0.1% agar solution and dispensed on 0.8% agar-gelled MS medium containing 2% sucrose. Plates were sealed with parafilm, moved to a cold room at 4 °C for 72 h to break dormancy and facilitate uniform seed germination, and then transferred to the culture room, under continuous illumination.

Hormone treatments of rice seedlings

For treatment with different hormones, 6-day-old light-grown rice seedlings were transferred to beakers containing solutions of indole-3-acetic acid (50 μM), epibrassinolide (10 μM), benzylaminopurine (50 μM), gibberellic acid (50 μM), 1-aminocyclopropane-1-carboxylic acid (50 μM), and ABA (50 μM) for 3 h. Mock-treated seedlings kept in water for 3 h served as the control.

RNA isolation

Total RNA was extracted using the RNeasy Plant mini kit (Qiagen, Hilden, Germany). To remove any genomic DNA contamination, the RNA samples were treated with RNase-free DNase I (Qiagen) according to the manufacturer's instructions. For each RNA sample, absorption at 260 nm was measured, and RNA concentration calculated as $A_{260} \times 40$ (μg·mL^{-1}) × dilution factor. The integrity of the RNA samples was monitored by agarose gel electrophoresis.

cDNA isolation, cloning and sequencing

The coding region of *OsTOP6A1*, *OsTOP6A2*, *OsTOP6A3*, and *OsTOP6B* were PCR amplified, using gene-specific primers and the first-strand cDNA synthesized from total RNA (2–3 μg) isolated from rice flowers using Stratascript™ Reverse Transcriptase (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The RT-PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) as per the manufacturer's instructions, and sequenced. The 5′-RACE and 3′-RACE were performed using SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) and BD Advantage™ 2 PCR Enzyme System (Clontech) following the manufacturer's instructions. After 30 or 35 cycles, the PCR products were examined by gel electrophoresis followed by sequencing.

Semi-quantitative RT-PCR analysis

The transcript levels of *OsTOP6A1* in different rice tissues, and of *OsTOP6A3* and *OsTOP6B* in transgenic *Arabidopsis* plants, were examined by RT-PCR with gene-specific primers using Titan™ One Tube RT-PCR System (Roche Molecular Biochemicals, Mannheim, Germany). The primer sequences are as follows: *OsTOP6A1*, 5′-ATGGCGGG GAGGGAGAAGAGG-3′ and 5′-CCTTGTTTGATCTTC TTGGGAATG-3′; *OsTOP6A3*, 5′-CTTAAGGTGGAGCT GAAGCTGCCGGTG-3′ and 5′-TCAAATCCAGTCCTGT TGCTGC-3′; *OsTOP6B*, 5′-CGAGGGCAATTATGGAGA CTCTGGGAG-3′ and 5′-TCAAGGAATAAATCTGAA CAC-3′. Expression of the *ACTIN* gene served as an internal control.

Real-time PCR expression analysis

The real-time PCR analysis was performed as described [57] using gene-specific primers. The primer sequences are listed in Table S3. The expression level of genes in different RNA samples was computed with respect to the internal standard genes, *UBQ5* or *ACTIN*, to normalize for variance in the quality of RNA and the amount of input cDNA. The relative expression of different genes in different RNA samples was assessed by the $\Delta\Delta C_T$ method (Applied Biosystems, Foster City, CA, USA).

Yeast two-hybrid assay

The MATCHMAKER GAL4 Two-hybrid System 3 (Clontech) was used to test possible protein–protein interaction between the proteins of interest. The complete ORFs of *OsTOP6A1*, *OsTOP6A2*, and *OsTOP6A3* were cloned into a TRP1-marked GAL4 activation domain construct vector, pGADT7 (*AD-TOP6A1*, *AD-TOP6A2* and *AD-TOP6A3*). The full-length ORF and PCR amplified partial *OsTOP6B* (pTOP6B, amino acids 1–420) were cloned into a LEU2-marked GAL4 DNA-binding domain construct vector, pGBKT7 (*BD-TOP6B* and *BD-pTOP6B*). Each of the fusion constructs *AD-TOP6A1–A3*, *BD-TOP6B* and *BD-pTOP6B* were transformed into *S. cerevisiae* strain Y187 for two-hybrid analysis. Protein–protein interaction was detected by the colony-lift filter assay using X-Gal staining according to the Clontech protocol. All the clones were also tested for self-activation. We rechecked all the positive yeast clones for the presence of the inserted genes by PCR using gene-specific primers.

Transient expression in onion epidermal cells

The complete ORFs of *OsTOP6A3* and *OsTOP6B* were PCR amplified, and fused translationally with the *GUS* gene in *Nco*I and *Bgl*II sites of the plasmid pCAMBIA 3301. The primer sequences used for PCR amplification are as follows: *OsTOP6A3*, 5′-GTACCATGGCGGAGAAGAA GCG-3′ and 5′-GATAGATCTAATCCAGTCCTGTTGC-3′; *OsTOP6B*, 5′-GTACCATGGACGACGACGCTG-3′ and 5′-GGAAGATCTAGGAATAAATCTGAACAC-3′ (restriction sites are underlined). The recombinant vectors were bombarded, expressed transiently into the onion epidermal cells, and analyzed histochemically as described [56].

Overexpression construct and floral dip transformation of *Arabidopsis*

The complete ORFs of *OsTOP6A3* and *OsTOP6B* were PCR amplified and cloned in *Xba*I/*Sac*I and *Xba*I/*Bam*HI restriction sites of modified pCAMBIA 2301 and pBI121 vectors, respectively. The primer sequences used for PCR amplification are as follows: *OsTOP6A3*, 5′-GACTCT AGAATGTCGGAGAAGAAGCGC-3′ and 5′-GACGAG CTCTCAAATCCAGTCCTGTTGC-3′; *OsTOP6B*, 5′-GTA TCTAGAATGGACGACGACGCTG-3′ and 5′-GTAGGA TCCTCAAGGAATAAATCTG-3′ (restriction sites are underlined). The modified pCAMBIA 2301 vector contains *OsTOP6A3* and *GUS* under independent 35S CaMV promoters. The resulting binary constructs were transformed chemically into *Agrobacterium* strain GV3101.

A. thaliana (ecotype Columbia) plants were transformed by the floral dip method [58]. The dipped plants were grown to maturity, and the seeds harvested. The T1 transgenic Columbia plants were selected in the presence of kanamycin (50 $\mu g \cdot mL^{-1}$) and further screened by PCR using gene-specific primers and the GUS assay. T2 seeds were collected from individual transformants (T1) and plated again on the selection medium to determine segregation ratios for kanamycin-resistant versus kanamycin-sensitive plants. The transgenes were concluded to be homozygous when no sensitive T4 seedlings segregated from seeds of T3 individual plants. All the analysis of overexpression lines was performed using plants (T4 and T5) homozygous for the transgene.

Root growth inhibition assays and stress treatments

The inhibition of root growth of wild-type and transgenic seedlings by ABA (Sigma, St Louis, MO, USA) was assayed as described [59]. Seeds of wild-type and transgenic lines were germinated on MS plates supplemented with various concentrations of ABA and NaCl for estimation of percentage germination. The number of germinated seeds (with fully emerged radicle tip) was expressed as the percentage of the total number of seeds plated (40–80). For dehydration stress, the seeds were germinated on 100, 200, and 300 mM mannitol, and the fresh weight was recorded after 8 days. All the experiments were repeated at least three times, and data in the form of the mean of three values with standard deviation are presented.

Microarray analysis

Total RNA was extracted from 10-day-old wild-type and transgenic seedlings grown under normal growth conditions using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The starting material was 5 μg total RNA. The microarray analysis was performed using one-cycle target labeling and control reagents (Affymetrix, Santa Clara, CA, USA). Probe preparation, hybridization to Affymetrix *Arabidopsis* genome arrays (ATH1-121501), washing, staining and scanning were carried out according to the manufacturer's instructions. Affymetrix GeneChip Operating Software (GCOS) 1.2.1 was used for washing, scanning, and first-order analysis. Sample quality was assessed by examination of 3′ to 5′ intensity ratios of poly(A) controls, hybridization controls and house-keeping genes. The image (.cel) files were imported into Avadis 3.3 prophetic software (Strandgenomics, Bangalore, India) for normalization by robust multichip average and differential expression analysis. A P value cutoff of < 0.01 was selected to identify the genes up-regulated or down-regulated more than twofold. To ensure the reproducibility of the results, two independent biological replicates of each sample were used for microarray analysis.

As the locus assignments and annotations of genes provided by Affymetrix contain errors, the information provided by TAIR (ftp://tairpub:tairpub@ftp.arabidopsis.org/home/tair/Microarrays/Affymetrix/) was used. The oligonucleotide sequences of the probes were mapped to the *Arabidopsis* transcript dataset from TAIR (release 6) using the BLASTN program with an e value cutoff $<$ 9.9e-6.

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Supplementary material

The following supplementary material is available online:

Fig. S1. Multiple alignments of the motifs 1–5 of TOP6 subunit A (A) and motifs 1–4 of TOP6 subunit B (B) proteins of rice with other homologs from different organisms.

Fig. S2. Schematic alignment of the position of introns in TOP6 subunits A and B homologs from rice and *Arabidopsis* in relation to their protein sequences.

Fig. S3. Phylogenetic analysis of the TOP6 subunit A homologs.

Table S1. *TOP6* genes in rice.

Table S2. List of up-regulated and down-regulated (>2-fold and $P<0.01$) genes in 35S::OsTOP6A3 (A3L5) and 35S::OsTOP6B (FL6BL11) transgenic plants.

Table S3. Primer sequences used for real time PCR expression analysis.

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