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PcTGD, a highly expressed gene in stem, is related to water stress in reed (*Phragmites communis* Trin.)

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Abstract **To compare differential gene expression among three ecotypes of reed (*Phragmites communis* Trin.), dune reed (DR), heavy-salt meadow reed (HSR) and light-salt meadow reed (LSR), mRNA transcripts were displayed by cDNA-AFLP (amplified fragment length polymorphisms). The result revealed that a relatively small number of genes are likely involved in adaptations of DR and HSR to stresses. A full-length cDNA encoding dTDP-D-glucose dehydratase gene (*PcTGD*) was subsequently cloned from DR. Northern blot analysis showed that it is highly expressed in stem as well as rhizoma of the three ecotypes. However, its expression in DR stem was much higher than that of the other two ecotypes. After the removal of water stress, the expression of *PcTGD* was significantly reduced, suggesting that it possibly plays a role in adaptation of DR to water stress through an osmotic regulation mechanism.**

Keywords: reed (*Phragmites communis* Trin.), water stress, dTDP-D-glucose dehydratase (*PcTGD*).

Land plants are sessile, and in order to survive they have to endure stresses such as drought, high salinity and extreme heat. Two major environmental factors, greatly reducing plant productivity, are drought and salinity[1]. In recent years, significant progress has been made to understand the molecular mechanisms underlying plant responses to the stresses[2—6]. However, in most studies a single environmental stress factor is applied before analyzing the consequent responses. In the natural habitats of plants adverse environmental factors are hardly present alone. For example, osmotic stress caused by drought in the summer is often accompanied by high-temperature stress[6], and the natural stresses are often progressive and continued. Under laboratory conditions, stresses induce cell damage and many stress-responsive genes do not actually contribute to tolerance because their induction likely reflects stress damage[3,5,7]. Therefore, impacts of natural and laboratory environmental stresses on plants and their respective responses are distinct. Analyzing

plant responses to natural stresses using naturally occurring variations is important for elucidating the mechanism of their adaptation to adverse environments[8,9].

Reed plants are hygrophilous of the *Gramineae* with a typical habitat of shallow fresh and brackish water area of the swamps, riversides and lakeside. However, they adapt well to diverse and adverse terrestrial habitats and have evolved various ecotypes, which exhibit stable genetic differences[10—12]. There are swamp reed (SR) and three terrestrial reed ecotypes (dune reed, DR; light-salt meadow reed, LSR; and heavy-salt meadow reed, HSR) growing in the desert regions of northwest China. In our previous studies, we have found some stable variations occurring within their morphological and physiological characteristics correlated with drought and saline habitats[13]. It was proposed that they have evolved specific adaptive mechanisms resulting from differential gene expression. Understanding these mechanisms requires the identification of differentially expressed genes. In this study, gene expression patterns of different reed ecotypes from the desert region were compared. The results indicated that adaptations of plants to adverse environments likely involve a small number of genes. A cDNA encoding dTDP-D-glucose dehydratase (*PcTGD*) gene was cloned from reed and its RNA transcripts are predominantly detected in stem with a possible role in water stress response.

1 Materials and methods

(i) Plant material. Leaves, stems and rhizoma of four reed ecotypes (SR, DR, LSR, HSR) were derived from the desert region in Linze County, Gansu Province, China. Sampling site was the same as described previously[13]. First and second fully expanded leaves and stems were harvested and frozen in liquid nitrogen before being stored at – 80℃ for analysis. To remove the stress, field plants were transferred to greenhouse and grown under no stress condition.

(ii) cDNA-AFLP. cDNA-AFLP was performed as described by Bachem et al.[14]. Total RNA of leaves and stems were extracted using a Qiagen Plant RNeasy Kit (Germany). First and second strand cDNA were synthesized using Superscript II RNase H⁻ Reverse Transcriptase (Gibco/ BRL) and SMART cDNA Library Construction Kit (Clontech), respectively. After *Taq*I/*Ase*I digestion and adaptor ligation, the cDNA products were pre-amplified and then diluted for cDNA-AFLP. Amplified products from different primer combinations were separated in a sequencing gel and exposed to X-ray film. Differentially expressed fragments cut from gel were cloned and sequenced. Homology search was done by the Blast program.

(iii) Isolation of a full-length *PcTGD* cDNA clone from dune reed. Among cloned dune reed stem-specific expressed fragments, a 640 bp one had a significant similarity to dTDP-D-glucose dehydratase gene of *Arabidopsis*. Based on its sequence, the 5′ and 3′ RACE (rapid amplification of cDNA ends) primers were designed as the following:

5′ RACE: 5′-ATGATTATCCTCCTCCCATG-3′

5′-AAGCAGTGGTATCAACGCAGAGT-3′

3′ RACE: 5′-AGGTGAATTTACCATGCTG-3′

5′-ATTCTAGAGGCCGAGGCGGCCGACATG-d-$(T)_{30}N_{-1}$ N-3′

N=A, G, C or T; N_{-1}=A, G or C.

A fragment of 650 bp was obtained by 5′ RACE. To isolate a full-length cDNA clone of this gene, a cDNA library was constructed from RNA of dune reed stem (SMART cDNA Library Construction Kit, Clontech) and screened using the 5′ RACE fragment as a probe labeled by α-^{32}P-dCTP (Prime-a-gene labeling system, Promega, USA). In total, 29 clones were identified and confirmed by nested PCR from about 1×10^5 phages. A clone with the largest insert was converted into plasmid according to the manufacturer's instruction and sequenced.

(iv) RNA expression analysis. Total RNAs were prepared as described above and 20 μg of total RNA from each sample was subjected to 1.0% formaldehyde denature agarose gel electrophoresis. After electrophoresis, the RNA was blotted to Hybond N⁺ nylon membrane (Amersham Pharmacia, USA) with 20×SSC. After pre-hybridization, the hybridization was conducted using radioactive probe according to the manufacturer's manual (Amersham Pharmacia, USA) and then the membrane was exposed to X-ray film (Fuji Photo Film Co., Japan).

2 Results

(i) cDNA-AFLP analyses of differentially expressed genes among reed ecotypes. To isolate genes related to adaptations to environmental stresses, differential gene expression pattern was compared among the three ecotypes of reed plants through cDNA-AFLP analysis of cDNA from their leaves and stems (fig. 1). In total, 138 pairs of primer combinations were used to perform selective amplification. To reveal the expression patterns, the displayed transcripts from randomly selected seven pairs of primer combinations were analyzed (table 1). The result showed that among the three ecotypes 77.2% bands were monomorphic, 2.2% bands specifically detected in SR, 8.1% in DR and 4.4% in HSR. 8.1% fragments were found only in DR/HSR but not in SR. Therefore, 2.2% fragments were SR-specific likely representing genes suppressed under water stress, and 20.6% fragments were DR/HSR-specific representing genes up-regulated under drought/salt stress. As the cDNA-AFLP templates were cut by restriction enzymes before pre-amplification, it is possible that some of the differences observed resulted from DNA polymorphisms occurring between the ecotypes. Nevertheless, these results indicated that the adap-

tations of the ecotypes to adverse habitats correlate to differential expression of a small number of genes.

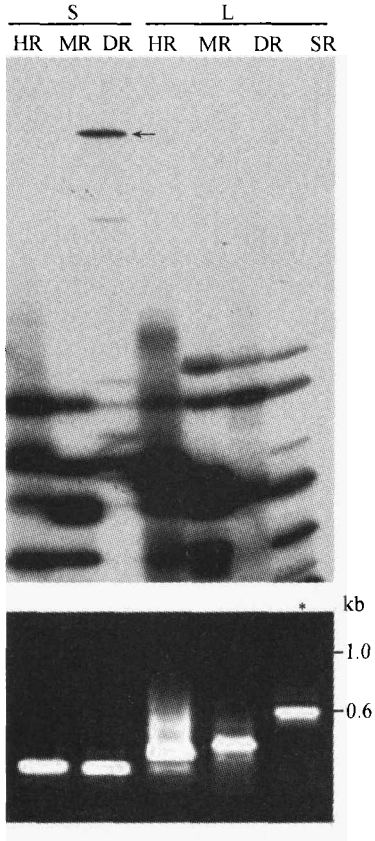

Fig. 1. cDNA-AFLP analysis of mRNA from reed leaves and stems. The upper panel shows a portion of the polyacrylamide gel containing separated cDNA-AFLP products. The arrow indicates the fragment representing *PcTGD*. SR, swamp reed; DR, dune reed; LSR, light-salt meadow reed; HSR, high-salt meadow reed. L, Leaf; S, stem. kb, kilobases. The lower panel represents the PCR products after reamplification of the cDNA-AFLP fragments separated by 1% agarose gel electrophoresis and stained by ethodium bromide. * is the lane containing the *PcTGD* fragment.

Table 1 Analysis of differentially expressed genes among the three reed ecotypes

Primer combination	Universal	SR-specific	DR-specific	HSR-specific	DR/HSR-specific	Subtotal
A_{14}/T_{17}	22	0	2	1	1	26
A_{14}/T_{12}	11	0	2	1	1	15
A_{18}/T_{12}	16	0	2	0	5	23
A_{18}/T_{4}	16	0	3	2	0	21
A_{18}/T_{6}	11	1	0	0	2	14
A_{18}/T_{17}	9	1	1	1	1	13
A_{14}/T_{13}	20	1	1	1	1	24
Total	105	3	11	6	11	136
%	77.2	2.2	8.1	4.4	8.1	100

To further characterize the differentially expressed genes, 118 differential fragments were cloned. Sequence analysis and BLAST search of 41 fragments indicated that 31(76%) showed no similarity to known genes in EMBL database, and 10 were highly homologous to known genes. A DR stem specific fragment (see fig. 1) showed high similarity with dTDP-D-glucose dehydratase gene and was named *PcTGD* (*Phragmites communis* dTDP-D-glucose dehydratase, EMBL accession no. AJ295156). Possible relationship between *PcTGD* and plant adaptation to adverse habitats was investigated because a previous study showed that *TGD* is related to stress in *Arabidopsis*[15].

(ii) Isolation of a *PcTGD* full-length cDNA clone. To isolate a cDNA corresponding to *PcTGD*, we screened the DR cDNA library with a 5′RACE product (about 650 bp) as probe (see section 1). A cDNA clone with an insert length of 1562 bp was identified and encoded an open reading frame (ORF) of 350 amino acids with a 238 bp 5′ non-encoding region and a 271 bp 3′ non-encoding region. The predicted protein has a molecular weight of 39.2 ku with pI=7.15 and high hydrophilicity. Comparison of the predicted protein and *TGD* sequences from other organisms is shown in fig. 2.

(iii) RNA expression analysis of *PcTGD*. To verify the expression pattern of *PcTGD* observed after cDNA-AFLP analysis, RNA gel blot analysis was performed. A 1.6 kb band was detected using the 5′RACE product as a probe (data not shown), indicating that the isolated cDNA clone represented a full-length copy of *PcTGD*. Northern analysis conducted using the full-length cDNA as probe demonstrated that *PcTGD* was expressed predominantly in stems with little expression in leaves and rhizoma (fig. 3), suggesting that *PcTGD* is highly expressed in stems. The expression of *PcTGD* in DR stems was threefold higher than that in SR; in addition, its expression in rhizoma of dune reed was also high with little expression in rhizoma of SR and HSR, indicating that it is positively correlated to water stress.

To further investigate the relationship between *PcTGD* and water stress, plants were transplanted and grown in greenhouse without stress for 1 month. Northern analysis showed that *PcTGD* expression remained high in stems and was not affected by the changing growth conditions (fig. 3). Compared with the plants in natural habitats, the expression level of *PcTGD* in SR stems did not change, but reduced in DR and HSR, especially reduced 3—4 folds in DR stems. These results further indicated that *PcTGD* expression is related to water stress. Furthermore, the expression of *PcTGD* in HSR was lower than that in SR under both natural habitat and greenhouse conditions (fig. 3), suggesting that its expression was suppressed under the salt stress condition. Phenotypes relevant to reed ecotypes were evolved during their long-term adaptation to adverse environment; therefore, it is likely that high level expression of *PcTGD* could not be recovered from the stress condition when plants were transplanted to a

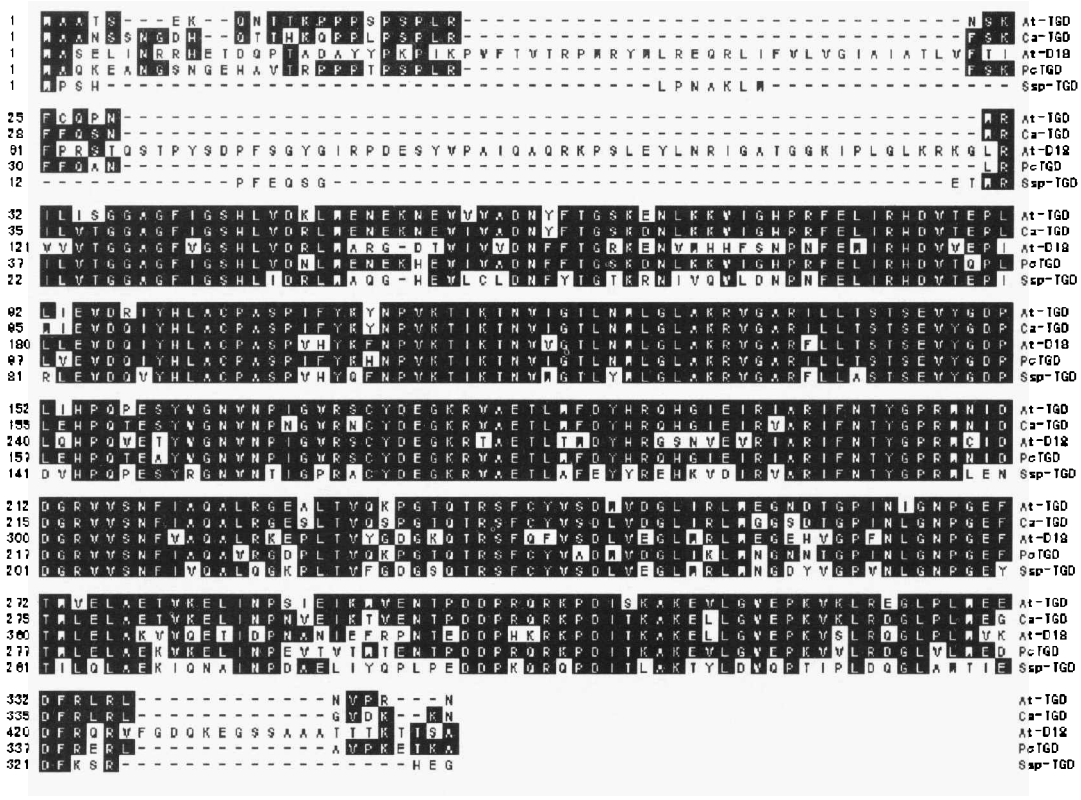

Fig. 2. Alignments of predicted TGD polypeptides. At, *Arabidopsis thaliana*; Ca, *Cicer arietinum*; AtD-18, a homologue of AtTGD; Ssp, *Synechocystis* sp. Pc, *Phragmites communis*. Identical amino acids are black boxed.

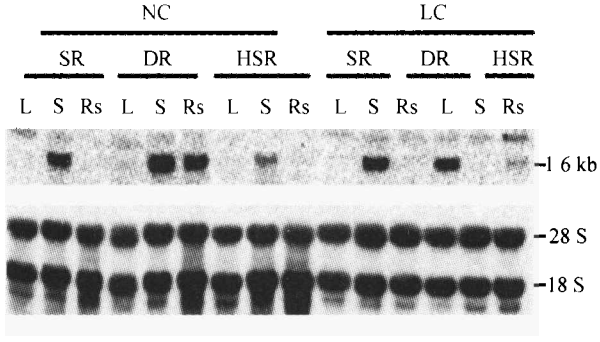

Fig. 3. Northern blot analysis of *PcTGD* under natural (NC) and greenhouse (LC) conditions. The lower panel is the Northern hybridization result of the same blot with rDNA as probe. SR, swamp reed; DR, dune reed; HSR, high-salt meadow reed. L, Leaf; S, stem; Rs, rhizoma; kb, kilobases.

favorite environment for a short term. This reflects a difference of plant gene regulation under the natural habitats and short-term man-made stress.

When RNA gel blot analysis was performed using the full-length *PcTGD* cDNA as probe, a weakly hybridizing mRNA of 2.0 kb was detected in addition to the *PcTGD* transcript (see fig. 3). This mRNA was not detected using the 5′RACE product as probe, indicating that it is a gene homologous to *PcTGD* in its 5′ or 3′ end region. Besides *TGD* from the other organisms, *PcTGD* showed a high similarity to UDP-glucose epimerase (identity 30%), especially to its 5′ end region. Therefore, it is highly likely that the 2.0 kb mRNA represents a UDP-glucose epimerase gene from reed.

3 Discussion

In this study, the differential expression patterns among three reed ecotypes naturally grown in the desert regions were compared. By cDNA-AFLP analyses, it was found that small number of the differentially expressed genes (15%—20%) are likely involved in reed adaptation to adverse environments. In a previous study, when plants of two unrelated lines of maize (*Zea mays* L.) and their hybrid were submitted to progressive water stress for 10 d, changes in leaf proteins were induced[6]. 78 proteins out of 413 showed a significant quantitative variation (increase or decrease), with 38 of them exhibiting a different expression in the two genotypes[6]. 19% proteins were related to water stress and 9.2% to genotype, similar to our results. The differences found in reeds were revealed at mRNA level, and some mRNA expression changes are not related to protein changes[16]. On the other hand, many genes (for example, *sos3*) important for salt tolerance are not induced by stress[17]. Nevertheless, the number of genes involved in plant adaptation to progressive water

stress is similar to that induced by natural water stress, suggesting that the number of genes related to water stress is not big.

The products of sugar metabolism are known to play roles in stress tolerance[2,18]. *PcTGD* is highly similar to *TGD* of *Arabidopsis*, *Cicer* and bacteria and, therefore a similar role in the biochemical metabolic pathway *in vivo*. In bacteria, *TGD* is involved in biosynthesis of secondary metabolites[19] and cell wall liposaccharide[20]. In plants, the function of *TGD* is unknown[14]. Plant *TGD* might be involved in monosaccharide metabolism similar to that in bacteria. In the three reed ecotypes, the accumulation of soluble sugars in DR leaf is higher than the other ecotypes and osmotic adjustment resulting from soluble sugars is critical for DR to adapt to water stress[20]. Because of a possible role of TGD in the monosaccharide metabolism, it is highly likely that it is related to the osmotic adjustment of DR adaptation to water stress. The expression of *PcTGD* in DR stems was much higher than that in the other ecotypes. Interestingly, the expression of *PcTGD* in stems of HSR suffering from the osmotic stress was suppressed, similar to the accumulation of soluble sugars in HSR[21]. These raise the possibility that soluble sugars play no critical roles in HSR adaptation to adverse environments.

With the respect of expression of *PcTGD* in organs of reed plants, its transcripts were mostly found in the stems and little in the leaves under both stressed and non-stressed conditions. When the plants were transplanted and grown in greenhouse, the transcript levels of *PcTGD* in SR stem remain unchanged but reduced in DR/HSR stems, especially in DR stems. Furthermore, in the three ecotypes the expression of *PcTGD* was detected in DR rhizoma but not in the other two rhizoma. Taken together, a correlation between *PcTGD* and water stress has been established. Most of water stress related/responsive genes found previously were isolated from leaves with expression in most organs, whereas *PcTGD* was predominantly expressed in the stems. It likely represents an example of a novel class of water stress induced genes that were predominantly expressed in the stems related to DR adaptation to water stress environment. *Arabidopsis TGD* (D-18) was identified after screening yeast expressing *Arabidopsis* cDNA for antioxidant genes and was shown to have the potential to confer yeast antioxidant tolerance[15]. But, its expression did not change under the oxidant stress condition and was expressed in the up-ground organs such as flowers, stems and roots before bolting[15]. Therefore, the expression pattern of *TGD* in reed is distinct from *Arabidopsis*. Further experiments are required to determine the specific cellular location of *PcTGD* expression and to confirm its role in water stress tolerance of reed plant.

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