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Gene cloning and characterization of a nonspecific lipid transfer protein from sheep grass, *Aneurolepidium chinense*, grown in semiarid area

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Introduction

To survive under and cope with the environmental stress, plants respond by physiological and biochemical changes. We used sheep grass (*Aneurolepidium chinense*), which is stress tolerant, to elucidate the genes related to the stress tolerance. We isolated a nonspecific lipid transfer protein gene (*Acltp*) as one of salt-stress related genes by differential display and cloned it. Non specific LTPs were encoded by small multigene families and generally possess activity which enhances the tranfer of several classes of phospholipids and/or glycolipids between cellular membranes. A variety of possible functions have been proposed for plant nsLTPs, including involvement in biosynthesis of epicuticular wax or cuticle (Sterk et al., 1991) and pathogen-defense (Molina et al., 1993). Moreover, several reports suggested that LTP must be related with environmental stress response (Torres-Shumann et al., 1992). We report here the relationship between environmental stress and nsLTP in sheep grass. Abscisic acid (ABA) seems an important factor for the response of *Acltp*. From the view point of biotic stress, we also confirmed the response of *Acltp* to salicylic acid. (SA).

Materials and methods

Sheep grass (*Aneurolepidium chinense*) was grown in a greenhouse and transferred into a phytotron under a natural light (25ºC for 13 h and 20ºC for 11 h cycle) for stress treatment except for heat and cold treatment. Plants were hydroponically grown in a culture medium (KH_2PO_4 , 2.5X10^{-3} M ; $MgSO_4$, 9.7x10^{-4} M; KNO_3, 5.0x10^{-3} M; $Ca(NO_3)_2$•$4H_2O$, 1.1x10^{-3} M; Fe-EDTA (EDTA•Na_2, 1.0x10^{-3} M; $FeSO_4$•$7H_2O$, 1.0x10^{-3} M), Micronutrients (H_3BO_3, 7.0x10^{-2} mM; $MnCl_2$•$4H_2O$, 6.9x10^{-3} mM; NaCl, 1.0x10^{-2} mM; $ZnSO_4$•$7H_2O$, 1.0x10^{-3} mM; $CuSO_4$•$5H_2O$, 5.0x10^{-4} mM; Na_2Mo_4•$2H_2O$, 2.7x10^{-3} mM)). Plants were subjected to a variety of stresses by addition of NaCl 300 mM, ABA (10^{-6}, 10^{-5}, 10^{-4} M), H_2O_2 (30 mM), sodium salicylate (1.5 mM), drought and cold (4°C) and heat (42°C) treatments. Plant leaves were harvested after each stress treatment for extracting RNA.

Total RNA was extracted from leaves of sheep grass plants grown for 9 d in a culture medium containing each 100-400 mM NaCl (The concentration was gradually increased in a stepwise manner by 100 mM for 2 d up to 300 mM and 400 mM for 3 d). A cDNA library was prepared from the poly(A)$^+$RNA using the cDNA synthesis kit (Amersham Pharmacia Biotech) and packaging kit of λphage using MaxPlax^{TM} Lambda Packaging Extract, following the procedures recommended by the manufacturers. The library was screened with the fragment of *Acltp* obtained by the differential display method (Shi et al., submitted) as a probe. DNA sequences were determined by dye-primer sequencing method on a DNA sequencer (model 373A:Perkin-Elemer).

Total RNA was isolated from sheep grass following as Nakamura et al. (1997). Northern hybridization was performed as described previously (Nakamura et al., 1997).

Results and discussion

We screened cDNA library constructed from sheep grass leaves using its *Acltp* DNA fragment obtained by differential display as a probe. We cloned *Acltp* homologous with barley *Hvltp* (Fig.1). The deduced amino acid sequence of sheep grass nsLTP was highly homologous with that of barley by 83. 4%. The N terminal sequence of 25 amino acids was considered to be a signal peptide targeting to secretory pathway.

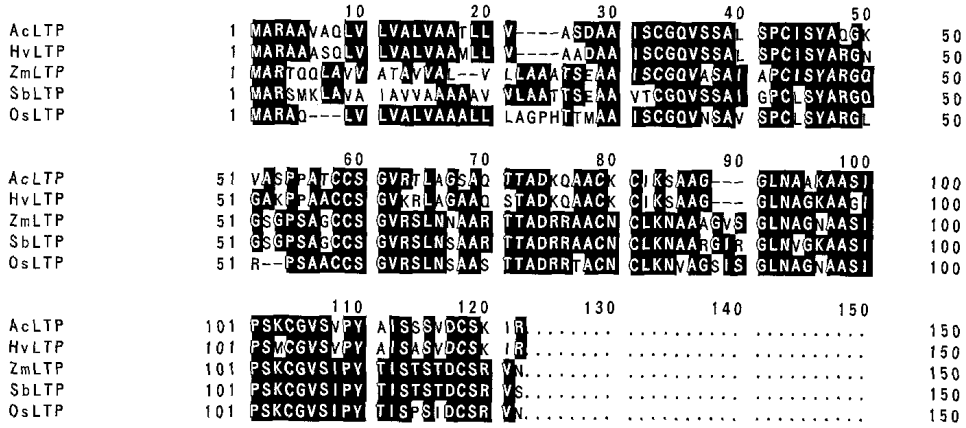

Fig. 1. Deduced amino acid sequence alignment of nsLTP from sheep grass compared with those from other plants. Eight Cys residues conserved in each LTPs. The sources and Genebank accession numbers of the other sequences are: AcLTP, *Aneurolepidium chinense* (sheep grass); HvLTP, *Hordeum Vulgare*, X68654; ZmLTP, *Zea maize*, J04176; SbLTP, *Sorghum bicolor*, X71668; OsLTP, *Oryza sativa*, Z23271.

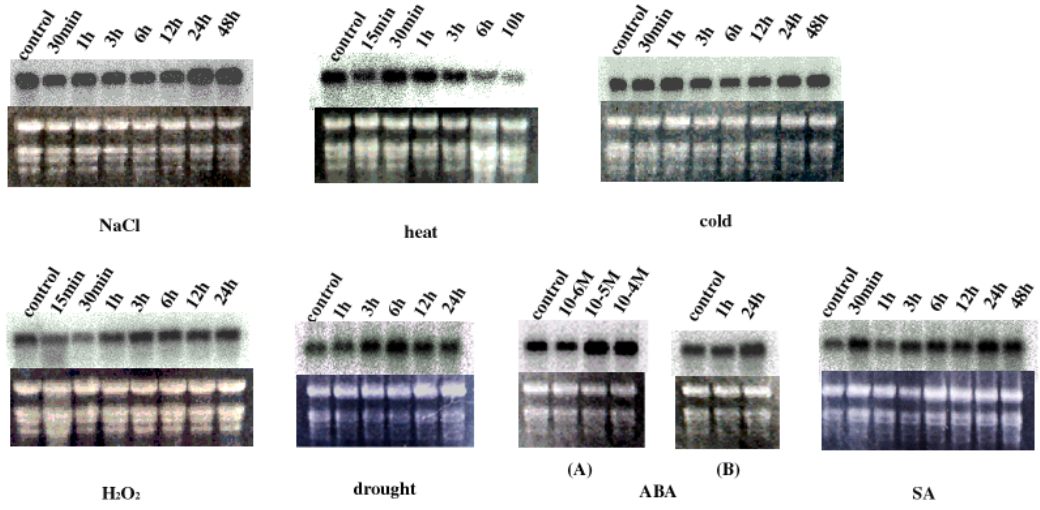

Fig. 2. Northern blot analysis for changes of *Acltp* transcript levels in sheep grass leaves under various stress treatments with NaCl, heat, cold, H_2O_2, drought, ABA and sodium salicylate. Total RNA was prepared from sheep grass leaves as described in Materials and Methods.

Northern blot analysis was done with 3'UTR as a probe (Fig. 2). We found that sheep grass *Acltp* is constitutively expressed under non-stress conditions. Under stress treatment with NaCl and cold, the expression of its mRNA level decreased transiently for 1-12 h, but then

recovered in 24 h and increased to some extent in 48 h. Under H_2O_2 treatment the expression recovered more rapidly than that under NaCl or cold treatment. By ABA, drought, and SA treatments, the expression of *Acltp* remarkably increased. In contrast, heat treatment decreased the expression of *Acltp* drastically after 6 h and the expression could not be seen at 24 h (Data not shown).

The expression of *Acltp* was clearly enhanced by drought, ABA, and SA. In *Arabidopsis*, at least 15 genes of LTP were identified and some of *ltp* genes were controlled by ABA, but others were independent on ABA (Vincent A et al., 2000). In sheep grass, we confirmed the increase of the level of mRNA under drought stress. In tabacco, the *ltp* gene was shown to be localized in guard cells and pavement cells in the epidermis and induced to some extent in drought-stressed guard cells (Smart LB et al., 2000). Sterk et al. (1991) have proposed a role for nsLTPs in cutin biosynthesis by effecting the transport of cutin monomers through the extracellular matrix mediated by ABA signal. Under NaCl and cold stresses, the expression of *Acltp* was recovered after 24 h. There is the possibility that the expression was modulated by ABA. Tentatively we hypothesize that nsLTP protect inner cells from the change of environment by strengthening cell wall of outer cells such as epidermal cells and guard cells. But under heat stress, we found that the *Acltp* was down-regulated after 3 h. SA enhanced the level of mRNA as well as ABA. SA is considered as a mediator of the systemic acquired resistance to pathogens (Raskin, 1992). So AcLTP also might take part in defence mechanisms.

In conclusion, sheep grass nsLTP seems to respond with both biotic and abiotic stress. However, *nsltp* is composed of multigene family, which include the genes whose expressions are pathogen-responsive or abiotic stress responsive. It is important to elucidate why the variously responsive types of gene exist. To analyse the function of all members of nsLTP will help us elucidate it.

Acknowledgments

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