# Molecular cloning and characterization of a gene encoding RING zinc finger ankyrin protein from drought-tolerant *Artemisia desertorum*

Xiuhong Yang, Chao Sun, Yuanlei Hu and Zhongping Lin*

*College of Life Sciences, National Key Laboratory of Protein Engineering and Plant Genetic Engineering, Peking University, Beijing 100871, People's Republic of China*

**Corresponding author (Fax, 86-10- 62759652; Email, linzp@pku.edu.cn)*

A RING zinc finger ankyrin protein gene, designated *AdZFP1*, was isolated from drought-tolerant *Artemisia desertorum* Spreng by mRNA differential display and RACE. Its cDNA was 1723 bp and encoded a putative protein of 445 amino acids with a predicted molecular mass of 47.9 kDa and an isoelectric point (pI) of 7.49. A typical C3HC4-type RING finger domain was found at the C-terminal region of the AdZFP1 protein, and several groups of ankyrin repeats were found at the N-terminal region. Alignments of amino acid sequence showed that AdZFP1 was 66% identical to the *Arabidopsis thaliana* putative RING zinc finger ankyrin protein AAN31869. Transcriptional analysis showed that *AdZFP1* was inducible under drought stress in root, stem and leaf of the plant. Semi-quantitative reverse-transcriptase-polymerase chain reaction (RT-PCR) analysis showed that the transcript of *AdZFP1* was strongly induced by exogenous abscisic acid (ABA) and also by salinity, cold and heat to some extent. Overexpression of the *AdZFP1* gene in transgenic tobacco enhanced their tolerance to drought stress.

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## 1. Introduction

Drought stress is one of the major environmental conditions that adversely affects plant growth and crop yield (Bartels and Nelson 1994). To deal with various environmental stresses, plants execute some physiological and metabolic responses (Bray 1993; Bohnert *et al* 1995). A number of plant transcription factors, such as AP2/EREBP, bZIP, WARKY, MYB and some zinc finger proteins, have been proved to be involved in plant response to environmental stress (Chen *et al* 2005).

Zinc finger proteins are among the most abundant proteins in eukaryotes, and play important roles in various cellular functions including transcriptional activation, regulation of apoptosis, protein folding and assembly (Laity *et al* 2001). The RING finger protein is a specialized type of zinc finger, and consists of 40–60 residues that bind two atoms of zinc (Freemont 1993). It is a bipartite asymmetrical motif which can be defined as Cys-$X_2$-Cys-$X_{(9–39)}$-Cys-$X_{(1–3)}$-His-$X_{(2–3)}$-Cys/His-$X_2$- Cys-$X_{(4–48)}$-Cys-$X_2$-Cys, where X is any amino acid (Borden and Freemont 1996). The RING finger motif was first identified about sixteen years ago as a human *Really interesting New gene* 1 (*RING1*) (Freemont *et al* 1991). Over the past few years, the number of proteins with RING fingers has grown enormously (Freemont 2000). More than 200 proteins from diverse eukaryotes have been found to contain this motif including some of the most important regulators of human disease and various cellular signalling pathways such as BRCA1 (a product of a breast cancer-associated gene) and Cb1, BNI-1, PML, p53-regulator MDM2 (the proto-oncogene products) (Freemont 2000; Jackson *et al* 2000). However, the specific function of the RING finger

**Keywords.** *Artemisia desertorum* Spreng; drought tolerance; mRNA differential display; RING zinc finger ankyrin protein; transgenic tobacco

Abbreviations used: ABA, abscisic acid; PEG, polyethylene glycol; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-polymerase chain reaction; RWC, relative water content

is unknown till now. Many proteins with RING fingers are thought to be involved in some aspects of transcriptional regulation and protein–protein interactions (Freemont 1993; Borden *et al* 1995; Satijn *et al* 1997). The latter studies have shown that many RING finger proteins have E3 ubiquitin ligase activities and they can specifically interact with E2 ubiquitin-conjugating enzymes, thus promoting ubiquitination (Joazeiro and Weissman 2000). Subsequent research has indicated that ubiquitination mediated by the RING finger E3 ubiquitin ligases is very important for the cell since the protein degradation must be highly selective for the cell to not cannibalize itself (Lee *et al* 2001).

Only a few genes encoding RING finger proteins, which were mainly identified in *Arabidopsis*, have been characterized in plants. The *Arabidopsis COP1* gene encoding a RING finger protein is a repressor of photomorphogenesis development and a light-regulated developmental molecular switch (McNellis *et al* 1994; McNellis *et al* 1996). The *Arabidopsis HOS1* gene, which encodes a novel protein with a RING finger motif near the N-terminal, has been demonstrated to function in low temperature signal transduction (Lee *et al* 2001).

*Artemisia desertorum* Spreng, belonging to the family Compositae, can survive under an extreme drought environment and is unique to China. Since it is highly capable of resisting a drought environment, it grows widely in the deserts in northwest China as an excellent sand-fixation plant species. In this paper, we cloned a new RING zinc finger ankyrin protein gene *AdZFP1* from drought-tolerant *Artemisia desertorum* Spreng by mRNA differential display and rapid amplification of cDNA ends (RACE), and introduced *AdZFP1* into tobacco through *Agrobacterium*-mediated transformation. The physiological responses of transgenic plants under drought stress were also investigated. The results showed that we had isolated a new RING zinc finger ankyrin protein gene *AdZFP1*, and the constitutive overexpression of the *AdZFP1* gene enhanced drought tolerance of transgenic tobacco plants.

## 2. Materials and methods

### 2.1 *Plant material and stress treatments*

Seeds of *Artemisia desertorum* Spreng, which were collected from Neimenggu Province, China, were sowed in a greenhouse with an 18 h photoperiod at 24ºC and 60% humidity. Two months after germination, the young seedlings were subjected to water stress treatment by withholding water for 3 weeks in the greenhouse. The leaves of *A. desertorum* with 60% relative water content (RWC) were harvested for RNA isolation and mRNA differential display. For further drought stress treatments, we subjected young *A. desertorum* plants to progressive drought by withholding water for 1, 2, 3 and 4 weeks in the greenhouse with an 18 h photoperiod at 24ºC and 60% humidity. The leaves of the plant were harvested for RNA isolation and northern blot analysis when the RWC was 80%, 70%, 60% and 50%, respectively. The root and stem of the *A. desertorum* plant were also harvested with 60% RWC. For further stress treatments, seeds of *A. desertorum* were grown on MS medium at 24ºC with a photoperiod of 18 h light/6 h dark. Salinity and exogenous abscisic acid (ABA) treatment were carried out by incubating plants on MS medium containing 200 mM NaCl and 100 $\mu$M ABA at 22ºC for 1, 3, 6, 12 and 24 h. ABA was dissolved in methanol, and 2.5% methanol was used as the control for ABA treatment. For low temperature treatment, plants were incubated at 4ºC for 1, 3, 6, 12 and 24 h. For heat treatment, plants were incubated in sealed flasks at 37ºC for 1, 3, 6, 12 and 24 h.

### 2.2 *RNA isolation and mRNA differential display*

The leaves of *A. desertorum* subjected to drought stress as well as controls were harvested for RNA isolation by using the Concert™ plant RNA Purification Reagent (Invitrogen, USA). The mRNA differential display was performed with the HIEROGLYPH™ mRNA Profile Kit System for Differential Display Analysis kit (Genomyx, USA.) according to the manufacturer's instructions. Four anchored primers (AP3, AP6, AP7, AP10) were used in combination with four 10 mer arbitrary primers (APR17, APR18, APR19, APR20). The cDNA fragments were electrophoretically separated on a 5.6% denatured polyacrylamide gel. The differential cDNA fragments were recovered from the sequencing gel and eluted. The elutes were used as templates for reamplification using the same sets of primer pairs. The reamplified fragments were purified from the agarose gel. The cDNA fragments of interest were cloned into the pGEM-T easy vector (Promega, USA) according to the manufacturer's instructions and then sequenced.

### 2.3 *5 RACE and RT-PCR*

The 5 -end of the cDNA was obtained with the 5 RACE system (Invitrogen, USA) according to the manufacturer's instructions. Three gene-specific primers were designed according to the partial sequence of the *AdZFP1* gene: GSP1 (5 -CGAGGTCTTGGTCAACTGTGCTAAC-3 ), GSP2 (5 -GATGGGTGCGGCTAGGGCAGAAG-3 ) and GSP3 (5 -GACCACAATCTTGAACCTCG-3 ). Total RNA from drought-treated plants was used as the template in the reverse transcription reaction. The PCR reaction was denatured at 94ºC for 2 min, and then subjected to 35 cycles of 94ºC denaturation for 30 s, 55ºC annealing for 30 s and 72ºC for 1.5 min, plus a final extension at 72ºC for 7 min.

Full-length cDNA was amplified by using RT-PCR with the primers *AdZFP1*S1 (5 -CGGGATCCATGGGACAGAATC TTAGC-3 ) and *AdZFP1*A1 (5 -CAGAGCTCCACAAAG CACAATACTCGCC-3 ) and cloned into the pGEM-T easy vector.

### 2.4 *Northern blot and semi-quantitative RT-PCR analysis*

Total RNA was separated by 1.0% formaldehyde agarose gel electrophoresis with 20 $\mu$g in each lane (Sambrook and Russell 2001) and transferred to Hybond-N$^+$ filters by capillary transfer. Northern blot was performed using the *AdZFP1* cDNA as a probe. To improve the detection sensitivity, an asymmetrical PCR approach (Sambrook and Russell 2001) was employed to label the probe. The labelling reaction mixture contained 2 $\mu$l 10 × PCR buffer, 0.4 $\mu$l dNTP (10 mM), 0.2 $\mu$l DIG-11-dUTP (1 mM), 0.5 $\mu$l primer *AdZFP1*S1 (1 $\mu$M), 2 $\mu$l primer *AdZFP1*A1 (5 $\mu$M), 0.5 $\mu$l cDNA template (50 ng/$\mu$l), 0.3 $\mu$l Taq DNA polymerase (5 U/$\mu$l), and 14.1 $\mu$l distilled water. The reaction was the same as the PCR procedure in section 2.3. After hybridization, the filters were washed with 2 × SSC, 0.1% SDS for 2 × 10 min at room temperature, and 0.1 × SSC, 0.1% SDS for 2 × 15 min at 68ºC. Detection analysis was performed with the DIG system under standard conditions according to the manufacturer's instructions (Roche, Germany).

Semi-quantitative RT-PCR analysis was conducted to investigate the expression pattern of *AdZFP1* under other stress conditions including high salinity, cold, heat and ABA. Total RNA was extracted from the leaves of *A. desertorum* subjected to the above stresses and treated with DNase I (Promega) to eliminate genomic DNA contamination. The first strand of cDNA was synthesized by using 1 $\mu$g of total RNA with SuperScript™ || Reverse Transcriptase (Promega) in a 20 $\mu$l reaction volume. Amplifications were performed at 94ºC for 5 min followed by 25 cycles of amplification (95ºC for 1 min, 55ºC for 1 min and 72ºC for 1.5 min). PCR products were separated on 1% agarose gels stained with ethidium bromide. The semi-quantitative RT-PCR reaction for the housekeeping actin gene using degenerate primers A1 (5 -ACT/A/CTTCTAC/TAAT/CGAGCTC/T/GCG-3 ) and A2 (5 -TCC/TTTCCTGATA/GTCA/G/CACATC-3 ) was performed under the same conditions as described above to estimate if equal amounts of RNA among the samples were used in RT-PCR.

### 2.5 *Vector construction and plant transformation*

Full-length *AdZFP1* was placed downstream of the CaMV35S promoter and ligated into the pCAMBIA3300 vector to construct the expression vector p3300-AdZFP1, as well as empty pCAMBIA3300 vector (as control), which were then introduced into *Agrobacterium tumefaciens* strain LBA4404 for tobacco transformation according to the procedure of Horsch *et al* (1985).

### 2.6 *PCR, Southern blot and northern blot analysis of transgenic tobacco plants*

When the transgenic tobacco plants were 4 weeks old, PCR amplification and Southern and northern blot analyses were performed to screen positive plants. Genomic DNA was extracted from the leaves of the tobacco plants by the cetyl trimethyl ammonium bromide (CTAB) method (Rogers and Bendich 1994) for PCR and Southern blot analysis. The primers in the PCR amplification were *AdZFP1*S1 and *AdZFP1*A1. The PCR reaction was the same as above.

Southern blot was performed using the *AdZFP1* cDNA as a probe. Aliquots of DNA (15 $\mu$g) were digested overnight at 37ºC with *Bam*HI, which was predicted to not cut within the *AdZFP1* cDNA. DNA was fractionated on 1.0% agarose gel and then transferred to Hybond-N$^+$ filters by capillary transfer. The hybridization was performed as the northern blot analysis described above.

Northern blot was performed using the *AdZFP1* cDNA as probe. Total RNA was extracted from the leaves of the transgenic tobacco plants by using Concert™ plant RNA Purfication Reagent (Invitrogen, USA). The transfer and hybridization was performed as the northern blot analysis described in section 2.4.

### 2.7 *Drought stress treatment and biological investigation of the transgenic tobacco plants*

Four-week-old *AdZFP1* transgenic tobacco plants as well as controls (with the empty pCAMBIA3300 vector) were then transplanted to equal amounts of soil in pots with equal amounts of water and grown in a greenhouse with an 18 h photoperiod at 24ºC and 60% humidity.

Before drought stress treatment, the transgenic tobacco plants and controls were cultivated under the same conditions and supplied fully with water. For drought stress treatment, we subjected young *AdZFP1* transgenic tobacco plants and controls (10-leaf stage) to progressive drought stress by withholding water for 1, 2 or 3 weeks in the greenhouse (24ºC, 60% humidity, and 18 h photoperiod). A gravimetric method was used to measure the soil water content. Samples were taken vertically from the soil in pots and weighed. After drying in an oven, the samples were weighed again. The percentage of moisture in the soil was calculated on the basis of dry weight. Soil water content was measured during the process of drought stress treatments. When the soil water content was 12% and 8%, the photosynthesis rate of the

3 upper leaves of the transgenic tobacco plants and controls was measured with the CI-301 CO2 gas analyser (USA).

The relative membrane permeability of the transgenic tobacco plants and controls was measured by the relative electric conductivity method (Liu *et al* 2000) when the soil water content was 12%.

Five positive transgenic tobacco lines proven by PCR, Southern and northern analysis, were chosen for the above treatments. Three repetitions were performed with 3 independent seedlings for each transgenic tobacco line and control in mensurating the photosynthesis rate and the relative membrane permeability.

## 3. Results

### 3.1 *Isolation and characterization of AdZFP1*

Differential display performed with 16 primer combinations resulted in the isolation of 34 fragments that presented different expressions between drought-treated *A. desertorum* and its control. Among these fragments, 4 fragments that showed significant positive signal on reverse northern blot were selected for further investigation. Sequence analysis of one of the 4 fragments revealed its homology to the RING zinc finger ankyrin protein gene isolated from *Arabidopsis*. The full-length cDNA, *AdZFP1* (GenBank accession number AY928808), was obtained by using 5 RACE followed by RT-PCR. The full-length *AdZFP1* cDNA consisted of 1723 nucleotides with an open reading frame of 1335 bp. The putative AdZFP1 protein contained 445 amino acids with a predicted molecular mass of 47.9 kDa and a pI of 7.49. A typical C3HC4-type RING finger domain was found at the C-terminal region of the AdZFP1 protein (figure 1). Several groups of ankyrin repeats were also found at the N-terminal region of the AdZFP1 protein. Alignments of the amino acid sequence showed that AdZFP1 was 66% identical to the *A. thaliana* putative RING zinc finger ankyrin protein AAN31869 whose function was unknown till now. Homology comparison in the NCBI GenBank database indicated the *AdZFP1* was perhaps a new member of the RING zinc finger family in plants.

### 3.2 *Expression profile of the AdZFP1 gene*

To investigate whether the expression of *AdZFP1* was regulated by multiple factors, its mRNA accumulation profile was determined under various abiotic stresses by northern blot and semi-quantitative RT-PCR. First of all, the *AdZFP1* gene was isolated from drought-treated *A. desertorum*, so it was important to further study the accumulation of *AdZFP1* transcripts during progressive drought stress when the RWC of leaves was 100% (as control), 80%, 70%, 60% and 50%. As a result, the *AdZFP1* transcripts increased rapidly within the water stress time course. The accumulation reached a maximum level when the RWC was 50%. Meanwhile, weak expression of *AdZFP1* was also detected in the control (figure 2).

The organ-specific expression pattern of *AdZFP1* was examined. Equal amounts of total RNA, isolated from the roots, leaves and stems, were subjected to northern blot analysis when the RWC was 60%. As a result, *AdZFP1* transcripts were detected in all tissues under drought stress treatment (figure 3).

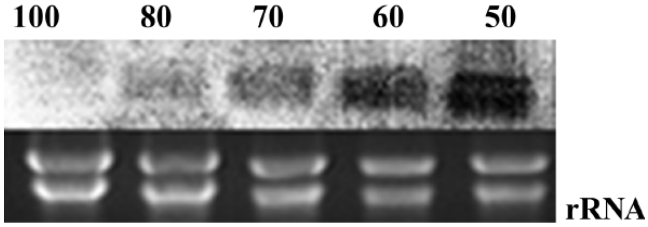


**Figure 2.** Northern blot analysis of *AdZFP1* transcripts when the relative water content of the leaves was 100%, 80%, 70%, 60% and 50% (upper panel), respectively. Equal loading of samples was confirmed by ribosomal RNA in the gel stained with ethidium bromide (lower panel).

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1     MGQNLSCGVK DDNGLFTAIQ YGDIEVVKHV MENDANFVVK KKTVYDRHSA LHIAAANGQI
61    EIVNLLLDKS SVNPDALNRR KQTPLMLAAM HGKIACVEKL IEAGANILMF DSLNGRTCLH
121   YAAYYGHSDC LETILSSART SHVAASWGFS RFVNIRDGKG ATPLHLAARQ RRPECVHILL
181   DSGALVCAST GGYGLPGSTP LHLAARGGSM DCVRELLAWG ADRLHRDASG RIPYAVALKH
241   NYGVCAALLN PSSAEPLVWP SPLKFISELN QDAKALLEQA LMEINRERER SILKGTGYSV
301   SSPSHSDATG MDDNISEASD SQLCCICFDQ LCAIEVQDCG HQMCAQCTLA LCCHDKPNPT
361   TSALAAPICP FCRSNIERLA VIKVKASTVD QGLDVFSSPK QRKSRRSINL SEGSSSFRGL
421   SGASFGKMVG RGSGRVSADL EWDKP
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**Figure 1.** Deduced amino acid sequence of *AdZFP1*. The RING finger motif is grey-shaded in the frame region.

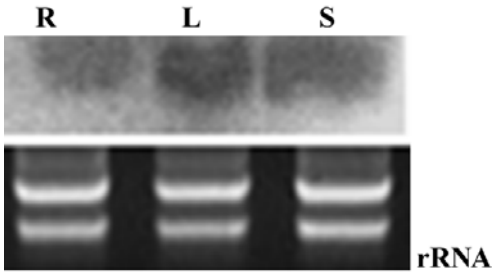


**Figure 3.** Northern blot analysis of *AdZFP1* transcripts in roots (R), leaves (L) and stem (S) of *A. desertorum* when the relative water content of the leaves was 60% (upper panel). Equal loading of samples was confirmed by ribosomal RNA in the gel stained with ethidium bromide (lower panel).

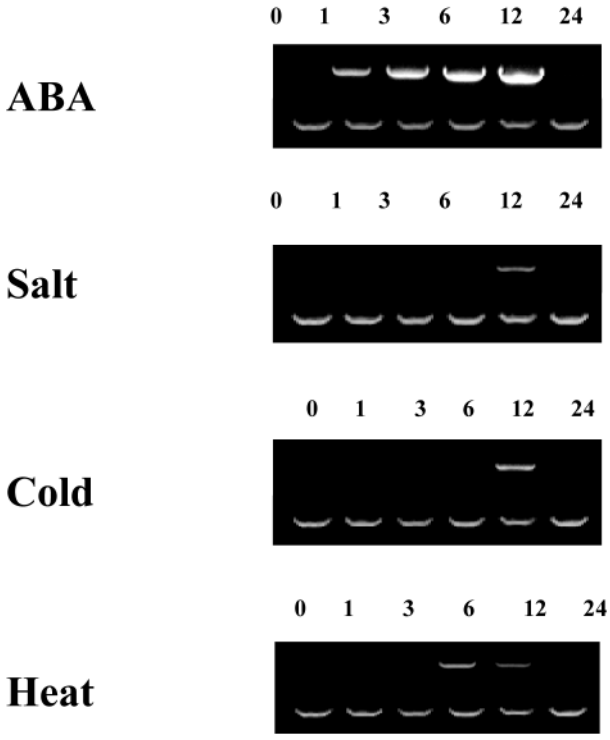


**Figure 4.** Expression profiles of *AdZFP1* under various treatments including ABA, salt, cold and heat. Total RNA (1 $\mu$g per sample) was isolated at 0, 1, 3, 6, 12 and 24 h after each treatment (upper panel, from left to right of each picture). The actin gene was used as the control to show the normalization of the amount of templates in the PCR reactions (lower panel of each picture).

Most drought-inducible genes have been found to be induced by exogenous ABA treatment (Bray 1997). The accumulation of *AdZFP1* transcripts treated with 100 $\mu$M ABA was studied by semi-quantitative RT-PCR at each time point (no obvious signal was found according to the results of northern blot). The *AdZFP1* transcripts accumulated when treated with exogenous ABA (figure 4). Obvious accumulation of *AdZFP1* transcripts were detected when treated after 3 h of exogenous ABA treatment. Within the time course of treatment, the transcription level showed a tendency to increase. The accumulation reached a maximum level after 12 h of treatment, while it decreased much after 24 h of treatment. Weak expression of *AdZFP1* was also detected in the control.

In order to study the expression of the *AdZFP1* gene under other environmental stresses such as salinity, cold and heat, semi-quantitative RT-PCR analysis was also used (no obvious signals were found on northern blot). The results showed that *AdZFP1* could have a response to all these stresses at a low level (figure 4). Maximum accumulation

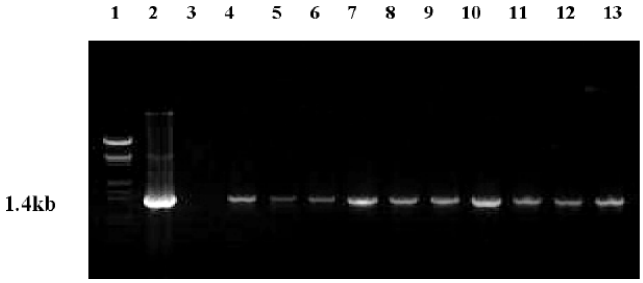


**Figure 5.** PCR identification of tobacco plants transformed with *AdZFP1*. 1, Marker ($\lambda$DNA/*Eco*RI+*Hin*dIII); 2, positive control (plasmid pGEM-T-AdZFP1); 3, tobacco transformed with empty vector pCAMBIA3300 (control); 4–13, independent transgenic tobacco lines.

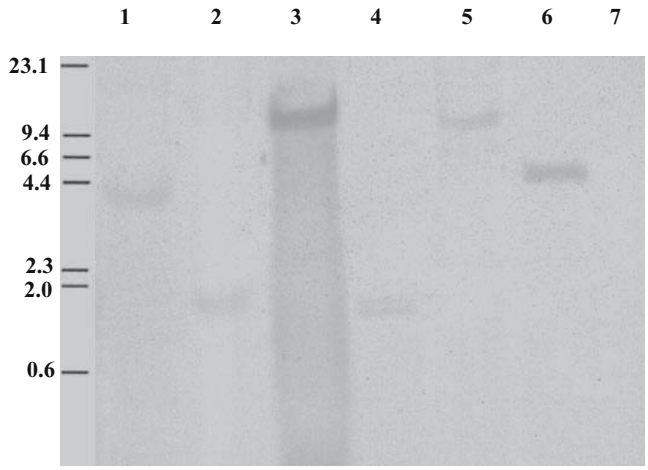


**Figure 6.** Southern blot analysis to confirm that the *AdZFP1* gene had integrated into the genome of transgenic tobacco lines. 1–6, different lines of *AdZFP1*-transformed tobacco plants; 7, tobacco transformed with empty vector pCAMBIA3300.

of the *AdZFP1* gene could be detected after 12 h salinity stress treatment and 12 h cold stress as well as 6 h heat stress treatment. A very slight expression of *AdZFP1* was detected in the control after each of the above treatments.

### 3.3 *Identification of transgenic tobacco plants by PCR amplification, Southern blot and northern blot analyses*

A binary expression vector was constructed with full-length *AdZFP1* driven by the 35 S promoter and used to transform tobacco W38. To determine if the *AdZFP1* gene had integrated into the genome of transgenic tobacco plants, PCR and Southern blot analysis were performed with the genomic DNA of transgenic tobacco plants. As shown in figure 5, the expected band (1.4 kb) was detected in 10 independent transgenic tobacco lines. Six of them were then randomly selected for further Southern blot and northern blot analysis (figures 6 and 7). The results showed that *AdZFP1* had integrated into the genome of transgenic tobacco plants and could be expressed normally in these transgenic plants.

### 3.4 *Biological index analysis of the transgenic tobacco plants under drought stress*

Compared with the controls transformed with the empty vector pCAMBIA3300, *AdZFP1*-transformed tobacco lines showed no obvious difference in phenotype when grown under normal conditions. Also, there were no phenotypic differences among the different *AdZFP1*-transformed tobacco lines during development, while under drought stress (the soil water content was 12%), phenotypic differences were observed among the *AdZFP1*-transformed tobacco lines and controls (figure 8). The control tobacco plants showed obvious wilting, while the transgenic tobacco plants looked well. The transgenic tobacco lines 2, 3, 4, 6 and 7 (figure 7) were chosen for subsequent biological analyses.

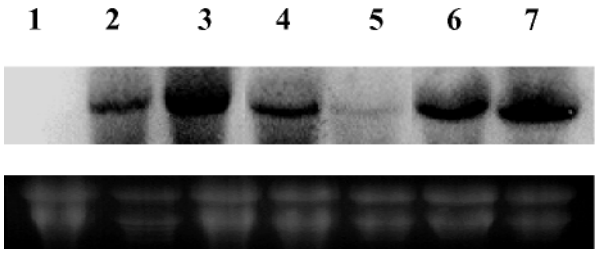


**Figure 7.** Northern blot analysis to confirm the expression of the *AdZFP1* gene in transgenic tobacco lines. 1, tobacco transformed with empty vector pCAMBIA3300 (control); 2–7, *AdZFP1*-transformed tobacco lines.

3.4.1 *Photosynthetic rate*: The transgenic tobacco lines 2, 3, 4, 6 and 7 (figure 7) and control tobacco plants were chosen for drought stress treatment. During progressive water deficit, the photosynthetic rate of the upper three leaves of each tobacco plant was measured using CI-301 $CO_2$ gas analyser (USA). When the soil water was enough, there was no obvious difference in the photosynthetic rate between the transgenic tobacco plants and controls. When the soil water content was 12%, the photosynthetic rate of all tobacco plants was reduced to some extent; the photosynthetic rate of the transgenic tobacco plants reduced by 33.8%, while that of the controls reduced by 57.0%, much more than that of the transgenic tobacco plants. When the soil water content was 8%, the photosynthetic rate of all plants was greatly reduced, but that of the transgenic tobacco plants reduced by 69.1%, and controls by 81.9%, more than that of the transgenic tobacco plants (figure 9). In other words, the transgenic tobacco plants could maintain a relatively high photosynthetic rate under progressive drought stress treatment.

After severe drought stress treatment, the transgenic tobacco plants and controls were supplied with enough water and began to recover from drought stress. The photosynthetic rate of the tobacco plants was then measured during their revival from the water stress. The photosynthetic rate of all the tobacco plants increased gradually following water supply. About 2.5 h after water was supplied, the photosynthetic rate of the transgenic tobacco plants reached the normal level; however, it took the controls 3.5 h to get to the normal photosynthetic rate (figure 10). Hence, the

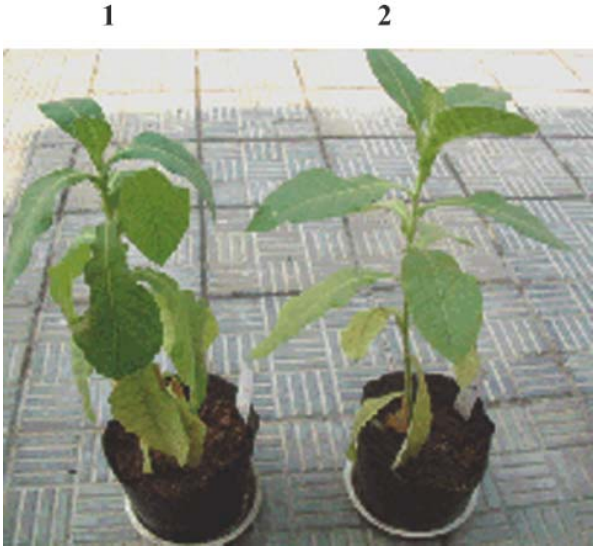


**Figure 8.** Photograph of transgenic and non-transgenic tobacco under drought stress treatment (the soil water content was 12%). 1, non-transgenic tobacco (control); 2, transgenic tobacco.

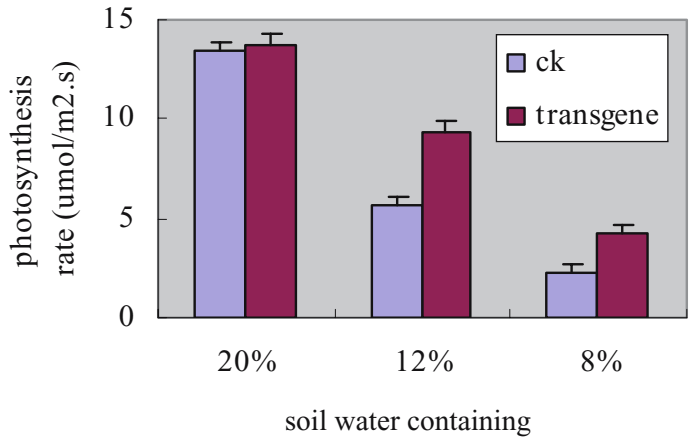


**Figure 9.** Photosynthetic rate of transgenic tobacco lines during progressive water deficit. Five positive transgenic tobacco lines 2, 3, 4, 6 and 7 (figure 7) were chosen for the treatment. Three repetitions were performed with 3 independent seedlings for each of them as well as the control. The upper three leaves of each tobacco plant were measured by the CI-301 $CO_2$ gas analyser. "ck" indicated the tobaccos transformed with empty vector pCIMBIA3300 (control).

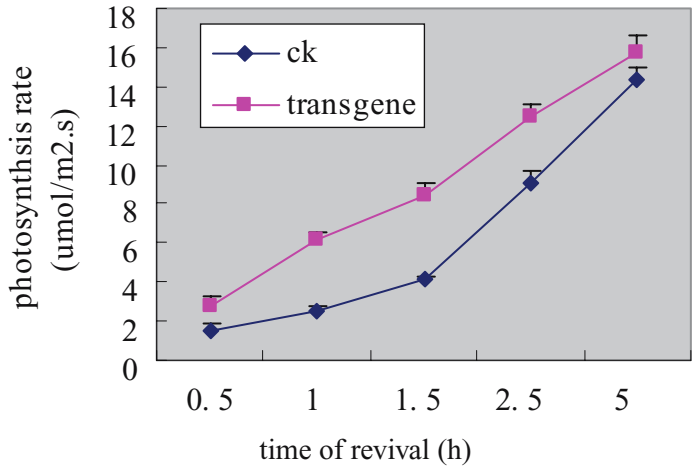


**Figure 10.** Photosynthetic rate of the transgenic tobacco lines during revival from water deficit. Five positive transgenic tobacco lines 2, 3, 4, 6 and 7 (figure 7) were chosen for the treatment. Three repetitions were performed with 3 independent seedlings for each of them as well as the control. The upper three leaves of each tobacco plant were measured by the CI-301 $CO_2$ gas analyser. "ck" indicates the tobaccos transformed with empty vector pCAMBIA3300 (control); 2, 3, 4, 6 and 7 indicated the five positive transgenic tobacco lines.

photosynthetic rate of the transgenic tobacco plants came back to normal sooner than that of the controls.

3.4.2 *Relative membrane permeability*: The membrane is the interface between the organelle and the environment for plants. It can receive and transfer information from the environment, and it can also respond to environmental stresses. The membrane permeability of plants would increase under environmental stresses, which does not benefit the normal biological function of the plant. We measured the membrane permeability of the transgenic tobacco lines 2, 3, 4, 6 and 7 (figure 7) and control tobacco plants under normal conditions (fully supplied with water) and drought stress (when the soil water content was 12%). The results revealed that there were no obvious differences in the membrane permeability between the transgenic tobacco plants and controls under normal conditions. Under drought stress, the membrane permeability of both the transgenic tobacco plants and controls increased, but the membrane permeability of the transgenic tobaccos increased by 8–29%, while that of the controls increased by 36–79% (figure 11). The results showed that the transgenic tobacco plants could

maintain a relatively low level of membrane permeability under drought stress, which might help the tobacco plants to cope with drought stress to some extent.

## 4. Discussion

Drought stress has adverse effects on plant growth and seed production, so it is very important to clone and characterize the drought stress-induced gene and find some useful genes to improve plant stress tolerance. Recent research suggests that transcription factors play an important role in signal transduction and gene expression under environmental stress (Chen *et al* 2002).

Signal transduction networks under environmental stress have been grouped into three major signalling types, of which type II signalling is involved in the production of stress-responsive proteins and functions in protection and damage repair, and may also be related to the ubiquitination process (Xiong *et al* 2002; Lee *et al* 2001). Many studies have indicted that the RING finger is involved in the ubiquitination pathway (Lorick *et al* 1999; Waterman *et al* 1999). The RING finger may act as E3 ubiquitin ligases and function in choosing specific targets for ubiquitination (Freemont 2000).

The RING finger motif was defined as a novel zinc-finger domain about sixteen years ago (Freemont *et al* 1991). Since then, several hundred proteins from diverse eukaryotes have been found to contain this motif (Lee *et al* 2001). Only a few genes containing the RING finger motif have been isolated from plants, and these have mainly been identified in *Arabidopsis*.

The *Arabidopsis HOS1* gene encoding a novel protein with a RING finger motif has been demonstrated to function in cold signal transduction by targeting a positive regulator of CBF/DREB1 expression for degradation (Lee *et al* 2001; Xiong *et al* 2002). This ubiquitination activity is dependent on the RING domain of *HOS1* (Yang *et al* 2000). The RING finger motif of *AdZFP1* shares a low identity with that of *HOS1*. Moreover, the RING finger motif of *HOS1* is located near the N-terminal, and that of *AdZFP1* is located near the C-terminal. Alignment of the RING finger domain of plants and animals indicates that the RING finger domain shares higher homology among animals including humans, and relative lower homology among plants (data were not shown). The difference between the RING finger proteins HOS1 and *AdZFP1* might implicate their differential functions in divergent signalling pathways. The RING finger domain of AdZFP1 with a similar function in signal or response to drought stress is worth further study.

Besides the C-terminal RING finger domain, the deduced amino acids of AdZFP1 also consisted of several groups of ankyrin repeats at the N-terminal. Ankyrin repeats often exist in functionally diverse proteins such as enzymes, toxins and transcription factors (Bennett and Chen 2001; Rubtsov and Lopina 2000). They mediate protein–protein interactions in very diverse protein families and are involved in signal transduction. Ankyrin repeats may occur in combination with other types of domains (Bork 1993; Sedgwick and Smerdon 1999). How the conserved domains of *AdZFP1* function and the relationship of the ankyrin repeats and RING finger protein in the signal transduction process need to be studied further.

More than half of the drought-inducible genes are also induced by exogenous ABA (Seki *et al* 2002; Chaves *et al* 2003), which plays an important role in the signal

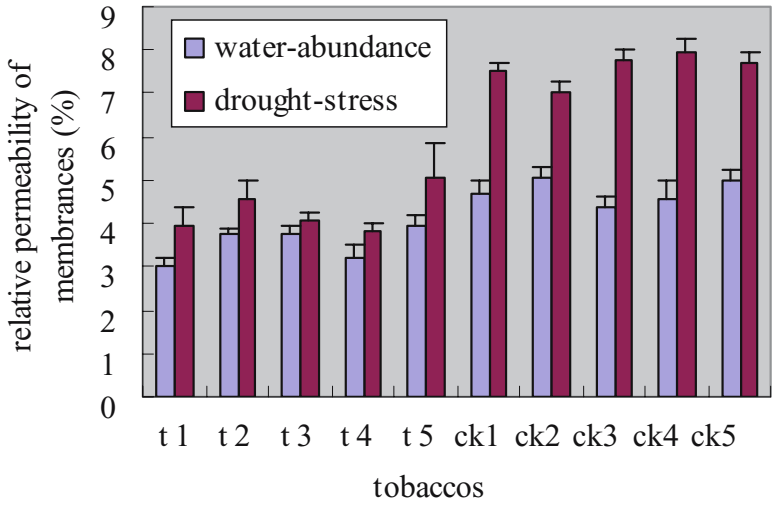


**Figure 11.** Relative membrane permeability of transgenic tobacco lines when treated with drought stress. t1–t5 represent transgenic tobacco lines 2, 3, 4, 6 and 7 (figure 7), respectively. "ck1–ck5" represent the tobaccos transformed with empty vector pCAMBIA3300 (control).

transduction pathways in response to abiotic stress (Leung and Girandat 1998). Semi-quantitative RT-PCR analysis showed that the expression of *AdZFP1* could be induced obviously by additional ABA, which indicated that *AdZFP1* might have a role in an ABA-dependent signal pathway. Previous research has indicated that most of the drought-inducible genes are also induced by high salinity, and 10% of the drought-inducible genes are induced by cold stress (Shinozaki *et al* 2003). Our results showed that *AdZFP1* responded to salinity, cold and heat stress at a lower level. The expression profile of the *AdZFP1* gene implied that *AdZFP1* might have complex roles in signalling pathways.

In this paper, a full-length cDNA clone *AdZFP1* encoding a RING finger ankyrin protein was isolated from drought-tolerant *A*. *desertorum*. Northern blot analysis revealed that the expression of *AdZFP1* was obviously induced by drought stress. Southern blot and northern blot analyses demonstrated that the *AdZFP1* gene had been inserted into the tobacco genome and was expressed normally in transgenic tobacco plants. Physiological analysis showed that transgenic tobacco plants containing the *AdZFP1* gene had an enhanced resistance to drought stress to some extent. We presumed that transgenic tobacco plants were more tolerant to drought stress because of the high photosynthetic rate and low level of membrane permeability under water deficit (figures 9, 11).

In conclusion, the RING finger ankyrin protein *AdZFP1* gene from *A*. *desertorum* has been cloned and characterized, and its functional investigation done in transgenic tobacco plants. Overexpression of the *AdZFP1* gene in tobacco plants enhanced their tolerance to drought stress. The results suggest that the RING finger ankyrin protein gene *AdZFP1* might be of importance in plant response to drought stress. Moreover, our results will be helpful in better understanding the molecular mechanisms of desert plant response to environmental factors as well as the role of the RING zinc finger in the signal transduction pathway under water-deficit conditions in plants.

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