Plant Mol Biol Rep (2009) 27:321–333
DOI 10.1007/s11105-008-0084-x

Enhanced Tolerance to Water Deficit and Salinity Stress in Transgenic *Lycium barbarum L.* Plants Ectopically Expressing *ATHK1*, an *Arabidopsis thaliana* Histidine Kinase Gene

Ni Chen · Yan Liu · Xin Liu · Juan Chai · Zhong Hu · Guangqin Guo · Heng Liu

Published online: 13 February 2009

Abstract *ATHK1* has been implicated in drought and salt tolerance in *Arabidopsis thaliana*. In this study, the full-length coding sequence of *ATHK1* was introduced into *Lycium barbarum* L. by *Agrobacterium* transformation. Our results indicated that the transgenic plants tolerated high concentrations of NaCl or water deprivation and exhibited faster recovery following re-watering compared to wild type plants. Salt- or water-stressed transgenic plants had higher relative water content, proline and soluble protein levels, and lower chlorophyll losses and membrane ion leakage. In addition, they showed higher capacity for antioxidative reactions reflected by reduced hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-), and lipid peroxide production and increased superoxide dismutase, catalase, and peroxidase activities. The *ATHK1* transcript, as shown by reverse transcription polymerase chain reaction, was more abundant under high than low osmolarity in transgenic plants. *ATHK1* therefore improved tolerance of *L. barbarum* to drought and salt stress.

Keywords *ATHK1* · Drought and salt stress · Drought and salt tolerance · Osmotic adjustment · Transgenic *Lycium barbarum*

N. Chen · Y. Liu · X. Liu · J. Chai · G. Guo · H. Liu (✉)
Institute of Cell Biology, Life Science School,
Lanzhou University,
Lanzhou 730000 Gansu, People's Republic of China
e-mail: hengliu@lzu.edu.cn

Z. Hu
Department of Biology, Shantou University,
Shantou 515063 Guangdong, People's Republic of China

Introduction

Lycium barbarum L. (also named *Fructus lycii* or wolfberry, family Solanaceae) is a perennial bush common to most areas of China, Europe, and the Mediterranean region. Its fruits have been used for centuries in China as a traditional herbal medicine and as a valuable nourishing tonic (Committee of Chinese Pharmacopoeia 1990). Recently, medical research has indicated that these fruits have many pharmacological functions such as improving vision, tonifying the liver and kidneys, nourishing blood function, resisting cancer and senescence, moisturizing the lungs, etc. (Chang and So 2007; Xie et al. 2001; Xu et al. 2000).

The worldwide increase in soil salinity and the scarcity of fresh water are two major abiotic stresses facing today's agriculture (Mahajan and Tuteja 2005). Drought and high salinity significantly reduce the yield and fruit quality of *L. barbarum* (Lin et al. 2007), but improving resistance to abiotic stress by traditional breeding technology in this perennial woody plant is a long-term endeavor. Modern transgenic approaches provide a more rapid means for overcoming drought and high salinity problems. Many effective protection systems exist in plants that enable them to perceive, respond to, and properly adapt to various stress signals (Bartels and Sunkar 2005; Bray 2004; Ma and Bohnert 2007; Maggio et al. 2006; Tran et al. 2007a; Yamaguchi-Shinozaki and Shinozaki 2006; Zhu 2002), and a variety of genes and gene products have been identified that involve responses to drought and high salinity stress (Ingram and Bartels 1996; Ma and Bohnert 2007; Shinozaki and Yamaguchi-Shinozaki 2007; Tayal et al. 2004). Genes expressed under conditions of abiotic stress are anticipated to promote cellular tolerance through protective functions in

the cytoplasm, such as alteration of cellular water potential to promote water uptake, control of cell membrane ion accumulation and retention, reduced production of reactive oxygen species (ROS) inside cells, and strengthening of ROS-scavenging enzymatic systems (RoyChoudhury et al. 2007; Yang et al. 2008).

Among these genes, *ATHK1* (At2g17820) has been identified as coding for a protein involved in osmotic stress perception under water deficit and salt stress, and its proposed product, a histidine containing phosphotransfer protein AHP2, has also been isolated in *Arabidopsis thaliana* (Urao et al. 2000, 2001; Wohlbach et al. 2008). *ATHK1* knockout mutants caused by T-DNA insertion in *Arabidopsis* indicate that *ATHK1* plays an important role upstream from MAPK in the osmotic stress signal transduction pathway (Hao et al. 2004). Moreover, gain-of-function and loss-of-function approaches show *ATHK1* to be a positive regulator of drought, salt stress, and abscisic acid (ABA) responses. Its transcripts accumulate rapidly when *A. thaliana* is exposed to external osmotic stress, suggesting an important function of *ATHK1* for the efficient sensing of environmental signals (Tran et al. 2007b).

Many characteristics of *L. barbarum*, such as its pest and disease resistance, growth habit, and fruit quality, could be manipulated by means of genetic transformation in a short period of time. Gene transfer technology through the *Agrobacterium*-mediated transformation of *L. barbarum* leaf explants and the subsequent regeneration of transgenic plants via somatic embryogenesis has been described (Du et al. 2006; Hu et al. 2002). However, few studies have focused on increasing the tolerance of *L. barbarum* to high soil salinity and water deficit through transgenic approaches. In this study, we generated transgenic plants expressing *ATHK1* to investigate the effect of this gene on drought and salt stress tolerance in *L. barbarum*.

The expression of *ATHK1* in *L. barbarum* resulted in enhanced tolerance to high salinity and water deficit. The *ATHK1* transgenic lines revealed several physiological and biochemical strategies for adaptation to drought stress conditions and salt-induced injury, which might be further characterized for better understanding of osmosensing in plants.

Material and Methods

Plant Material and Growth Conditions

A. thaliana and *L. barbarum* seeds were disinfected in 0.1% (*w*/*v*) $HgCl_2$ for 10 min, then rinsed five times with sterile distilled water. Surface-sterilized *Arabidopsis* seeds were grown on Murashige and Skoog's (MS) agar medium at 22°C with a 16-h day/8-h night cycle for RNA extraction.

Surface-sterilized *L. barbarum* seeds were germinated on 1/2 MS medium (pH 5.8) under a 16-h light/8-h dark photoperiod at 22°C for transformation. For various physiological experiments, the seeds were sown aseptically on 1/2 MS medium supplemented with 100 mg/L kanamycin. After 20 days, they were transplanted to plastic pots containing a mixture of vermiculite and turf soil (1:1, by volume) in a greenhouse under 16-h light/8-h dark cycles at 22°C. The plants were watered every 2 days.

Generation and Screening of Transgenic *L. barbarum* Plants

Total RNAs were extracted from *Arabidopsis* as described by the Arabidopsis Laboratory Manual (2004). The full-length coding sequences (CDS) of *ATHK1* was amplified by PrimeScript™ RT-PCR kit (TaKaRa) using primers H1 (5′CCGCTCGAGATGCGAGGAGATAGCTTCT CA3′) and H2 (5′CCGCTCGAGTCAAGCGGACAATGAA GTTTG3′). The resulting fragment was then cloned into pKYL71 at the *Xho*I site to yield 2×P_{35S}-*ATHK1*, and the correct inserted direction was confirmed by sequencing.

The pKYL71 plasmid containing correct direction 2×P_{35S}-*ATHK1* and the empty pKYL71 plasmid was transformed into *L. barbarum* by *Agrobacterium tumefaciens* LBA4404 with the leaf-disc method (Liu et al. 2007). Transgenic plants were transferred into soil.

Southern hybridization and reverse transcription polymerase chain reaction (RT-PCR) were then used to further screen transgenic plants containing *ATHK1* gene. RT-PCR was carried out by using the two primers (H3: 5′CAGAGG AAACGGAAGTGATG 3′, H4: 5′AGATAGACTTGTAG TTGAGGGA 3′). The extracted DNA was digested with *Hin*dIII for 2 h, then separated in 1% agarose gel and transferred onto nylon membrane for southern hybridization. Radioactive P_{32}-labeled probe was generated by PCR from *ATHK1* cDNA with the same pair of RT-PCR primers. Hybridization and exposure were carried out according to standard methods (Sambrook and Russell 2000).

Drought, Osmotic and Salt Stress Treatments

For drought and osmotic stress treatment tests, healthy wild-type (WT) and transgenic *L. barbarum* plants, grown under a normal watering regime for 8 weeks, were divided into three groups, each containing ten individual WT and transgenic plants of similar size and age. The first group of plants was treated with 30% (*w*/*w*) polyethylene glycol (PEG) 6000 for 24, 48, and 72 h to simulate osmotic stress then was photographed. The second group was subjected to drought stress by omitting watering, followed by rehydration after

30 days. Observation and photos were taken at 15 and 30 days, then again 10 days after re-watering. The third group served as a control and was watered every 2 days.

For salinity resistance experiments, healthy and green 8 weeks WT and transgenic plants of similar size and age were divided into six groups, which were respectively treated with different concentration neutral salt solutions containing 0, 0.3%, 0.6%, 0.9%, 1.2%, 1.5% NaCl (NaCl weight: dry soil weight) for salt tolerance observations.

Evaluation of detached leaf water loss ratio, relative water content (RWC), and ion leakage ratio of cell membranes

The rate of water loss from excised leaves was measured as described by Jenks et al. (1994) with minor modifications. The same sized leaf sections were excised from transgenic and control lines, then submerged in distilled water, and soaked for 2 h. Excess water was then removed with filter paper, and the leaf sections were then weighed (g_0). After drying in an oven at 70°C, they were weighed again each 1 h (g_t). Leaf water loss was calculated as follows: fresh weight loss (%)=$(g_0-g_t)/g_0\times100$. Relative water content was carried out according to the method of Brini et al. (2007). Membrane damage was measured and was assayed by measuring ion leakage from leaf discs according to Rizhsky et al. (2002).

Measurement of MDA, H_2O_2, O_2^-, CAT, SOD, and POD

The comparative rates of lipid peroxidation were assayed from the leaves of control and stress-treated *L. barbarum* seedlings by determining the levels of malondialdehyde (MDA; Velikova et al. 2000). H_2O_2 levels from leaf tissues of control and salt- or drought-treated *L. barbarum* seedlings were determined by the method of Velikova et al. (2000). O_2^- production rate was measured using the hydroxylamine reaction of Elstner and Heupel (1976) with some modifications. Fresh seedling tissues were ground with 600 μl 65 mmol/L phosphate buffer (PB) and filtered. Filtrate was centrifuged at 5,000×*g* for 10 min and then 300 μl of the upper phase was transferred to a new centrifuge tube, followed by the addition of 270 μl PB and 30 μl 10 mmol/L hydroxylamine chloride and incubation for 20 min. Then, 600 μl 17 mmol/L *p*-aminobenzene sulfonic acid and 600 μl 7 mmol/L *a*-naphthylamine were added to each tube. After 20 min of incubation at 25°C, an equal volume of *n*-butyl alcohol was added and the mixture was centrifuged again. The absorbance of the supernatant layer was read at 580 nm against a PB blank.

Catalase (CAT; EC1.11.1.6) activity was assayed by monitoring the decomposition of H_2O_2 at 240 nm (E= 40 mM^{-1} cm^{-1}) according to the method of Aebi et al. (1974). The reaction mixture contained the appropriate extract in 50 mM potassium PB (pH 7.0), and 10 mM H_2O_2 was added to initiate the reaction. One unit of CAT activity was defined as the amount of protein that catalyzed the decomposition of 1 μmol H_2O_2 per minute. The activity of superoxide dismutase (SOD; EC1.15.1.1) was measured according to the protocol of Giannopolitis and Ries (1977) and was monitored as the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). A unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition in the reduction of NBT at 560 nm and was expressed per milligram protein. Peroxidase (POD; EC1.11.1.7) activity was measured according to the method of Velikova et al. (2000) with some modifications. The oxidation of guaiacol was measured by the increase in absorbance at 470 nm over 1 min. The reaction mixture contained 600 μl guaiacol 1% (*w*/*v*) aqueous solution, 2.8 ml of 10 mM PBS (pH 7.0), and 0.1 ml enzyme extract. The reaction was initiated with 150 μl of 100 mM H_2O_2.

Determination of Free Proline, Total Chlorophyll, and Soluble Protein

Determination of free proline content was performed as described by Bates et al. (1973) in leaves of similar age and size, as proline content can vary depending on leaf size and plant age. The total chlorophyll content of healthy and fully expanded leaves (of similar age and size) from untreated or 0.9% NaCl and drought-treated plants was determined spectrophotometrically according to the method described by Arnon (1949) with some modifications. Chlorophyll was extracted by soaking leaves in dimethyl sulfoxide solution for 48 h. Absorbance of extracts was measured at 635 and 645 nm with a spectrophotometer. Soluble protein was determined by the Bradford method using bovine serum albumin as a standard (Bradford 1976).

ATHK1 Gene Expression Analysis of Transgenic *L. barbarum* Under Different Stresses

Eight-week-old transgenic *L. barbarum* seedlings were treated with either 0.9% NaCl for 5 days, drought for 15 days, PEG 6000 for 48 h, or 100 μM ABA. For each plant, semiquantitative RT was performed using 2 μg total RNA per reaction (Weigel and Glazebrook 2004).

Measurement of Endogenous ABA

Endogenous hormones was extracted according to Walker-Simmons (2005), purified using Sep-Park C_{18} cartridges (Waters Co., USA), and stored at −20°C before detection. ABA was detected with an ELISA kit (Nanjing Agricultural University) according to the manufacturer's instructions.

Statistical Analysis

All experiments were performed in triplicate with ten plants of similar age and size per treatment. Data represent the means of three experiments (±SD).

Results

Stable Expression of *ATHK1* Full-Length CDS in *L. barbarum*

To improve *L. barbarum* responses to water deficit and salt stress, full-length CDS of *ATHK1* (Fig. 1a) was cloned by RT-PCR and expressed in plasmid pKYL71 with the 2×CaMV 35S promoter. The empty pKYL71 plasmid and the pKYL71 plasmid with the resulting fragment were then transformed into *L. barbarum* by *A. tumefaciens* with the leaf-disk method. The pKYL71 vector contains the *NPT-II* gene (conferring resistance to kanamycin) as a selecting marker (Fig. 1b), and more than ten transgenic plants were obtained. Kanamycin-resistant transformants were confirmed as having the *ATHK1* CDS integrated successfully by Southern hybridization (Fig. 1d) and that it could be stably expressed by RT-PCR (Fig. 1c).

In this experiment, WT plants and transgenic plants bearing empty vectors had no apparent differences in their morphology after 30 days dehydration stress or treatment with 0.9% NaCl, so WT plants were used as controls for the remainder of the experiments.

Enhanced Tolerance of Transgenic Plants to Drought and Osmotic Stress

Transgenic *Lycium* plants showed no apparent differences in their morphology compared to WT (Fig. 2a).

In order to observe drought stress tolerance of transgenic plants, we compared WT lines with the *ATHK1* transgenic lines over a prolonged period of drought treatment. The leaves of the transgenic plants retained their green color

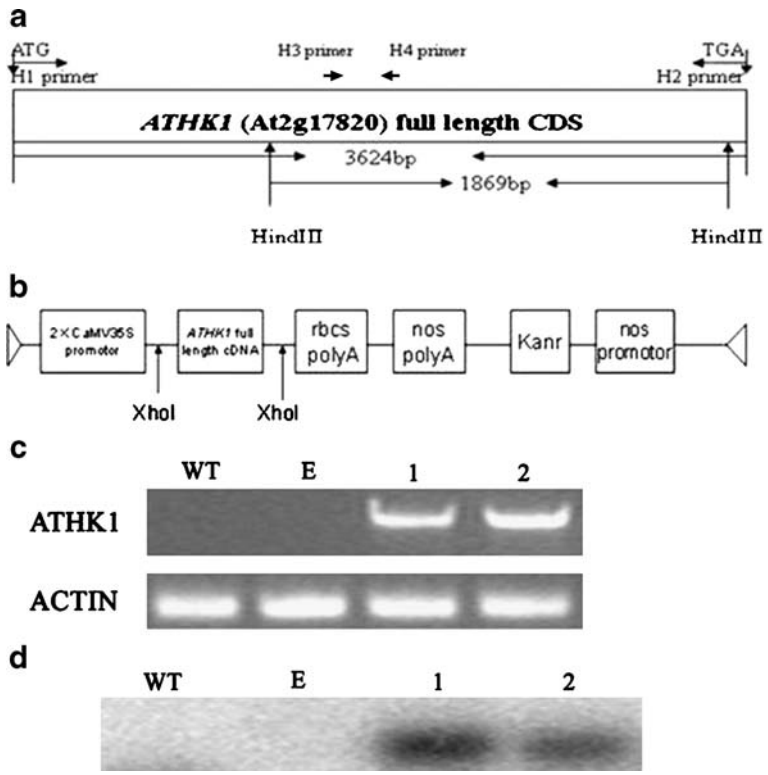

Fig. 1 Confirmation and analysis of transgenic *L. barbarum* expressing *ATHK1*. **a** Diagram of *ATHK1* full-length CDS. H1 and H2 primers used for cloning of full-length CDS of *ATHK1*; H3 and H4 primers used for Southern blotting hybridization and RT-PCR analyses. **b** Schematic map of the pKYL71 construct used for *L. barbarum* transformation. *ATHK1* full-length CDS was inserted between the 2× CaMV 35S promoter and terminator rbcs polyA at *Xho*I site. **c** RT-PCR analysis showing the expression of *ATHK1* in WT, transgenic lines, and transgenic lines bearing empty vector using specific primers. ~430-bp fragment indicated that *ATHK1* was constitutively expressed in transgenic *L. barbarum*. *WT* non-transformed WT plants, *E* transgenic plants bearing empty vector. *Lanes 1* and *2* independently transformed ATHK1 plants. ACTIN: RNA was used as an internal template control. **d** Southern blotting analysis to confirm the presence of *ATHK1*. ~1,870-bp hybridization band showed that *ATHK1* were successfully integrated into the *L. barbarum* genome. DNA was digested with *Hin*dIII, separated, and hybridized to H3, H4 probe. *WT* non-transformed WT lines used as control. *E* transgenic plants bearing empty vector. *Lanes 1* and *2* DNA from different ATHK1 transformed plants

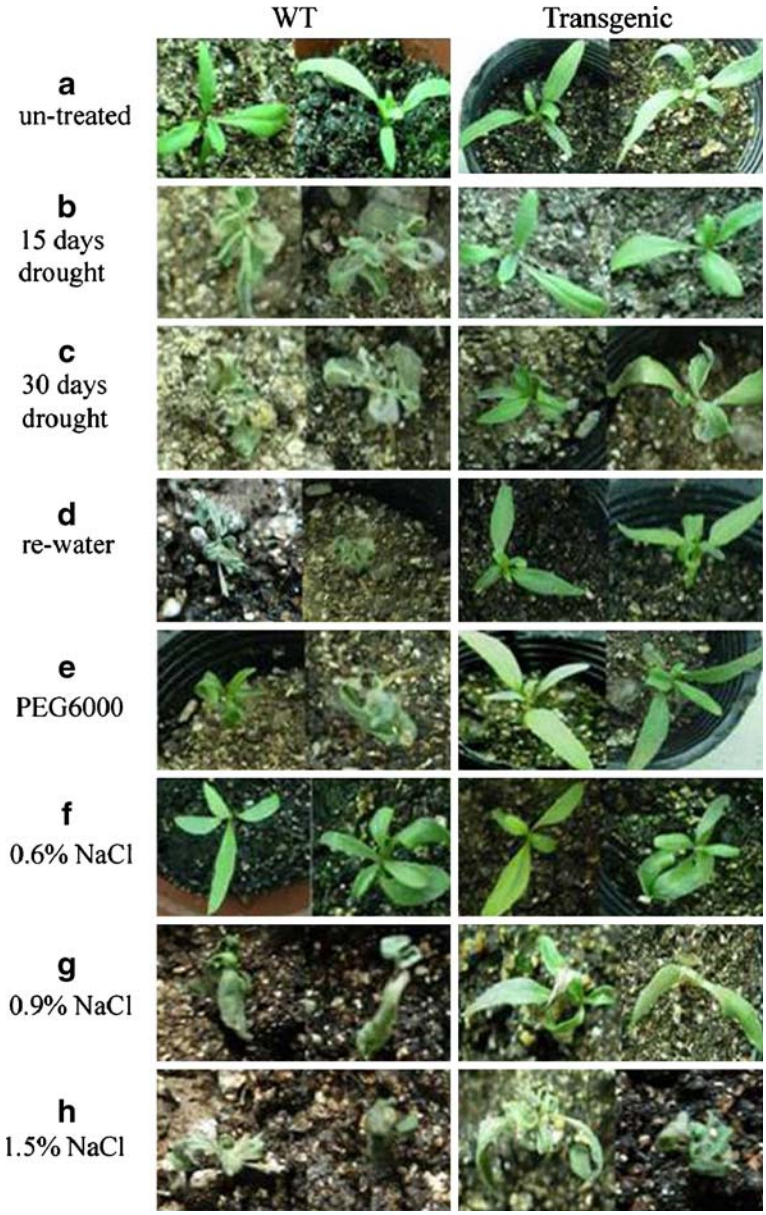

Fig. 2 WT (untransformed) and transgenic *L. barbarum* L. plants under drought and salt stresses. **a** WT plants and transgenic lines were grown on soil in standard conditions. Plants were watered by capillarity every 2 days. **b** After 15 days of water deficit stress. **c** After 30 days of water deficit stress. **d** Ten days after re-watering, following 30 days drought treatment. **e** After 30% PEG6000 for 72 h. **f** After 10 days of 0.6% NaCl treatment. **g** After 10 days of 0.9% NaCl treatment. **h** After 10 days of 1.5% NaCl treatment

during the stress period, while the leaves of WT plants lost their green color and began to crinkle within 15 days of water deprivation (Fig. 2b). After 30 days of deprivation, the leaves of transgenic lines showed a slight wilting, while WT leaves became totally dried up and substantially faded (Fig. 2c). After 30 days dehydration stress, the transgenic plants recovered their vigor within 10 days of re-watering, while the WT plants started to die (Fig. 2d).

To study the response to osmotic stress in *ATHK1* transformed plants, we also treated transgenic and WT plants with 30% PEG6000, a commonly used inducer of water deficit or osmotic stress (Behera et al. 2003). None of the plants showed any apparent differences after 24 h, but the leaves of the WT plants began to wither after 48 h and were obviously faded by 72 h compared to the transgenic plants (Fig. 2e). This result showed that WT plants were more sensitive to osmotic stress than were transgenic lines.

Increased Tolerance to Salt Stress in Transgenic Plants

To test whether *ATHK1* expression could enhance salt tolerance, 8-week seedlings were subjected to 0, 0.3%, 0.6%, 0.9%, 1.2%, and 1.5% NaCl and plant survival was monitored daily. No obvious difference was observed between the transformed and WT seedlings when treated with low salt concentration (less than 0.6% NaCl; Fig. 2f). The toxic effects of higher salt concentration (above 0.9%) were delayed and obviously attenuated in *ATHK1* transgenic lines. After 10 days at 0.9% and 1.2% NaCl, the *ATHK1* transgenic lines continued to grow, albeit at a slower rate,

whereas control lines (non-transgenic) exhibited chlorosis and died (Fig. 2g). When transformed and WT lines were subjected to 1.5% NaCl for 10 days, transformed plants began to wither and etiolate, whereas the WT plants died (Fig. 2h).

RWC and Water Loss Ratio of Detached Leaves After Drought and Salt Treatments

No noticeable differences between the WT and transgenic lines were seen in the well-watered normal growth condition. However, after 15 days of withholding water, differences in plant water status between the transgenic and WT lines became evident. RWC in the stressed condition declined from the initial value of 80.7% to 45.9% in the WT plants, but was maintained at about 82.6% in the transgenic lines (Fig. 3a). The RWC in leaf tissues of WT plants declined from the initial 80.5% value to 37.3% after 10 days at 0.9% NaCl. In the transgenic plants, RWC was unaltered (83.4%) after 10 days of salt treatment (Fig. 3b). The rate of water loss of detached WT leaves over 12 h was faster than that for transgenic lines under standard water and soil conditions (Fig. 3c).

Ion Leakage Ratio of Cell Membrane after Drought and Salt Treatments

In WT leaves, the ion leakage ratio was 50% higher than control values after 15 days of water deficit and 43% higher after 10 days of 0.9% NaCl treatment. In contrast, transgenic plants showed no apparent changes in ion leakage from the untreated condition (Fig. 4a).

Effect of Water Deficit, Osmotic, and Salt Stresses on Proline Content

Under well-watered conditions, the concentration of free proline in leaves of transgenic plants was similar to that of WT plants. After 15 days of drought stress, proline content in transgenic lines was four times higher than WT plants. After 72 h of 30% PEG6000 treatment, the free proline content of transgenic plants was substantially higher than that of WT plants (Fig. 4b).

When exposed to increasing NaCl concentration, the free proline content of transgenic plants showed marked increases compared to the WT plants, with the greatest increase noted at 0.9% NaCl (Fig. 4c).

Chlorophyll Loss after Water Deficit, Osmotic, and Salt Stresses

Total chlorophyll content in leaf discs was significantly decreased by 15 and 30 days of drought. However, at the same water stress, the chlorophyll loss in transgenic plants was significantly less noticeable than that in the WT plants. Salinity (0.9% NaCl) reduced the chlorophyll content by 29.5% after 5 days in WT plants and by 46.5% after 10 days. The transgenic plants suffered a much lower chlorophyll loss, with the maximum loss after 5 days of salt stress being 10% and 13% after 10 days. (Fig. 4d)

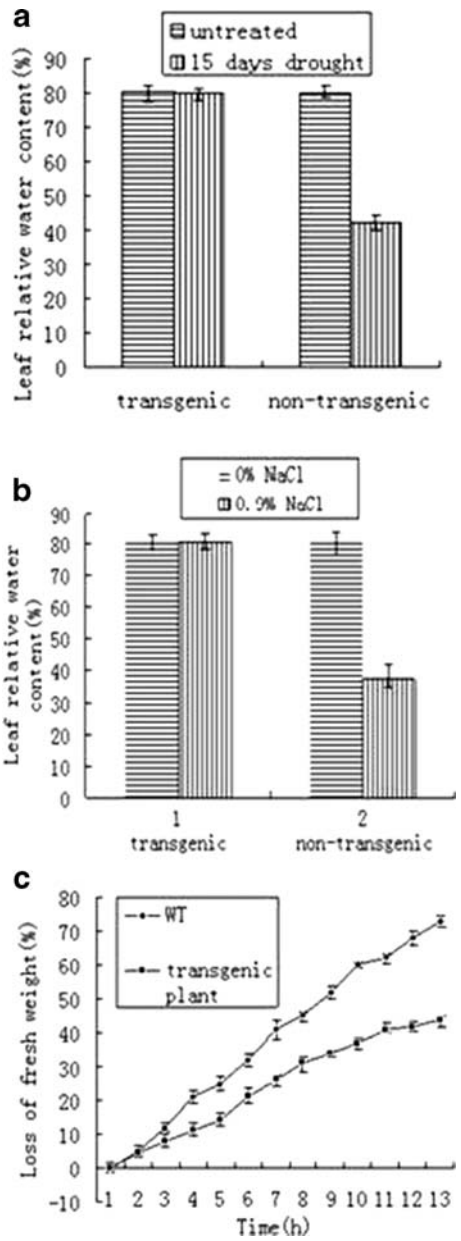

Fig. 3 RWC of WT and transgenic *L. barbarum* after 15 days of water deficit (**a**) or after 10 days salt stress of 0.9% NaCl (**b**) and the water loss rate of excised leaves of *ATHK1* transgenic and WT plants under normal condition (**c**). Results are the mean ± SD of three individual measurements

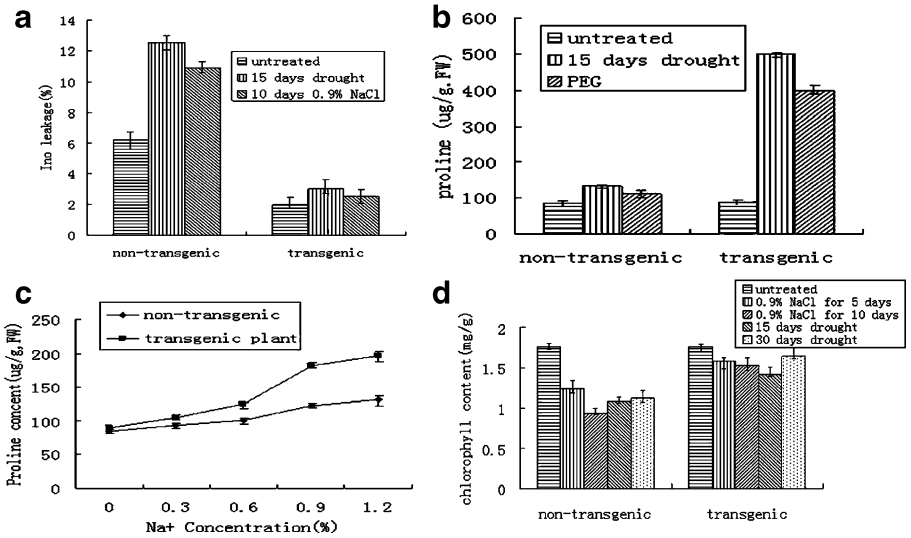

Fig. 4 Effects of drought and salinity stresses on *L. barbarum*. Cell membrane ion leakage ratio of leaves between *ATHK1* transgenic and WT plants after 15 days water deficit or 10 days of 0.9% NaCl treatment (**a**). Proline content of WT and *ATHK1* transgenic plants after 15 days drought or 30% PEG6000 for 72 h (**b**) or after increasing NaCl concentrations for 10 days (**c**). Chlorophyll contents were determined for leaf disks of WT and transgenic plants under 0.9% NaCl for 5 or 10 days and after water deficit for 15 or 30 days (**d**). Untreated *L. barbarum* seedlings served as experimental control. Values are the mean ± SD (*bar*) for three observations (n=3)

Lipid Peroxidation During Water Deficit, Osmotic, and Salt Stresses

MDA concentration, used as a measure of lipid peroxidation, was similar in WT and transgenic plants grown in the standard growth conditions. Under saline conditions, the WT plants showed maximum rise in MDA concentration (almost 2.1 times above the basal level) after 10 days of salt treatment. In contrast, the transgenic plants showed only a slight increase in MDA levels (0.2 times) over basal levels (Fig. 5a).

After 15 days of water deficit, or after 30% PEG6000 treatment for 72 h, a relatively strong increase in MDA production was observed in WT plants, but only a slight increase was seen in the transgenic lines (Fig. 5b).

H_2O_2 and O_2^- Levels Under Drought and Salt Stress

H_2O_2 levels in WT plants increased 4.2 times after 15 days of water deficit compared to well-watered counterparts. In contrast, the transgenic plants showed only a 1.18-fold increase in H_2O_2 levels over untreated plants. In general, the overall increases in H_2O_2 after water deficit stress were comparatively lower in transgenic lines than in the WT lines (Fig. 5c). The endogenous O_2^- production rate was the same in both transgenic and non-transgenic plants under normal conditions. In response to drought stress, O_2^- production rate markedly increased (4.9-fold) in WT plants but was only slightly increased in transgenic plants after 15 days or water deficit (Fig. 5d).

Salt stress caused a marked increase in the level of H_2O_2 and production rate of O_2^- of WT plants with increases in NaCl concentration, while these increased much more slowly in transgenic lines (Figs. 5e, f). The increases in H_2O_2 content and O_2^- production rate were most evident at 0.9% NaCl.

Antioxidative Defense Systems in *L. barbarum* Under Water Deficit and NaCl Stress

There were almost no differences in the activities of these three enzymes among transgenic and WT lines under normal conditions. However, when plants were treated with different concentrations of NaCl, the activities of CAT, SOD, and POD in WT seedlings declined rapidly, except at 0.3% NaCl where a slight increase in SOD or CAT activities was seen. At the 0.9% NaCl concentration, almost no CAT enzyme activity was detected, while SOD and POD activities were considerably lowered in WT plants. In transgenic lines, on the other hand, the activities of these three enzymes were increased in response to various concentrations of salt (Table 1).

SOD, POD, and CAT activity in WT plants declined after 15 days of drought stress, and all enzyme activity had almost disappeared after 30 days of drought. However, at the same water conditions, SOD, POD, and CAT activities in the *ATHK1* transgenic lines had increased and were significantly higher throughout the water deficit period (Table 2).

Effect of Drought and Salinity Stress on Soluble Protein Levels

Protein content decreased markedly in response to both water and salt stress in leaves of WT lines compared to

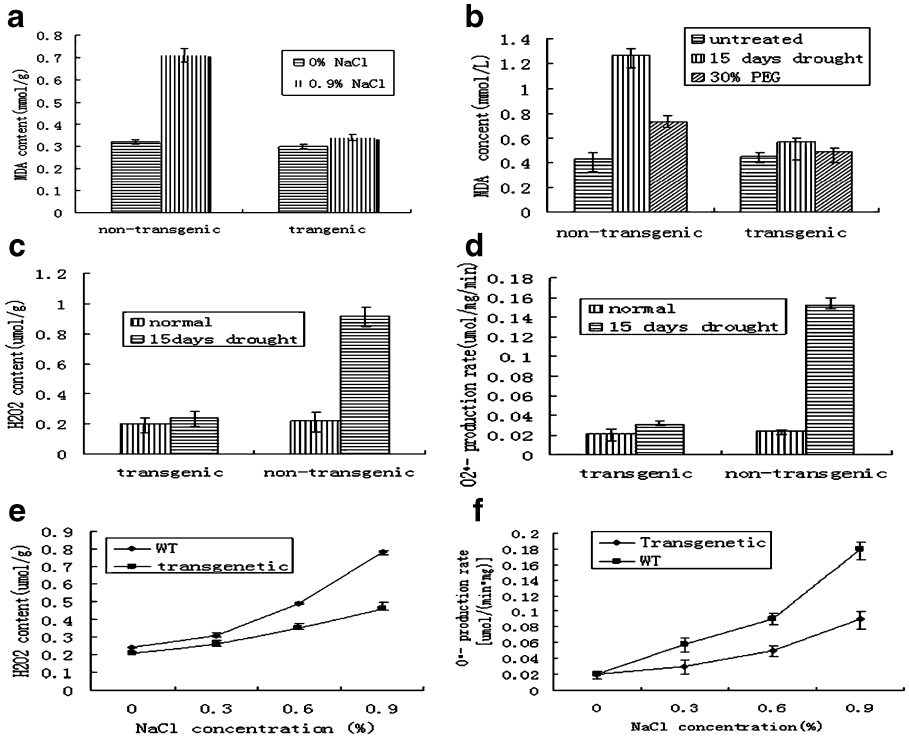

Fig. 5 MDA concentration and ROS (H_2O_2 and O^{2-}) activity as an index of lipid peroxidation and oxidative injury in WT and *ATHK1* transgenic *L. barbarum* lines in response to salinity and drought stresses. MDA content in response to 0.9% NaCl salinity (**a**) and 15 days drought (**b**). H_2O_2 content (**c**) and O^{2-} production rate (**d**) under 15-day drought stress conditions. Effect of increasing NaCl concentrations on H_2O_2 content (**e**) and rate of O^{2-} production (**f**). The data represent the means of three observations (n=3). The *vertical bar at the top* represents standard error in each case

unstressed controls. In transformed lines, soluble protein decreases were less than those in WT under the same stress conditions (Fig. 6a).

Increased *ATHK1* gene expression in transgenic *L. barbarum* plants under drought, salt, and osmotic stresses

To examine the levels of *ATHK1* gene expression in transgenic *L. barbarum* plants under various stresses and hormone treatments, ten independent transgenic lines were subjected to semiquantitative RT-PCR. As shown in Fig. 7, transcript level of the ATHK1 gene was higher under 0.9% NaCl after 15 days drought or following PEG6000 compared to levels from transgenic plants growing in normal watering and soil conditions. Expression of the *ATHK1* gene induced by ABA was not increased obviously in transgenic lines.

Endogenous ABA Content of Transgenic *L. barbarum* Plants Under Drought and Salt Stress

ABA content increased sharply during a 30-day drought treatment and then began to decrease (Fig. 6b). Under salt stress, endogenous ABA content initially increased reached a maximum at 0.9% NaCl. ABA levels declined at higher NaCl concentrations (1.2% and 1.5%; Fig. 6c).

Discussion

Plants have developed multiple physiological and biochemical systems which enable them to tolerate abiotic environmental stresses (Ma et al. 2006; Amtmann et al. 2005). Osmotic stress is one of the major factors reducing plant growth and productivity (Boyer 1982), and it occurs under drought as well as high salinity and low temperature conditions. The change in osmotic potential in cells caused by water loss and high salinity triggers various molecular responses in plants (Ma and Bohnert 2007; Xiong and Zhu 2002). Overexpression of *ATHK1* using its native promoter increased *ATHK1* expression under dehydration or salinity stress and consequently improved the drought and salt tolerance of transgenic *A. thaliana* plants (Tran et al. 2007b). In this paper, a full-length CDS of *ATHK1* in *Arabidopsis*, with a major open reading frame of 3,621 bp and that encoded a polypeptide of 1,207 amino acids with a predicted molecular weight of 135 kDa, was isolated by RT-PCR. This cDNA was successfully transformed into *L.*

Table 1 The effect of increasing NaCl concentrations on the activities of CAT, SOD, and POD in WT and transgenic *L. barbarum* L plants

salt concentration (%)	Protective enzymes activity					
	SOD ($U \cdot g^{-1} \cdot h^{-1}$ FW)		POD ($U \cdot g \cdot g^{-1}$ FW)		CAT ($U \cdot g^{-1} \cdot min^{-1}$ FW)	
	Transgenic	WT (control)	Transgenic	WT (control)	Transgenic	WT (control)
0	179.3±7.1	176.9±5.8	149±5.2	142±5.8	24.27±0.6	24.21±0.2
0.3	189.3±4.7	182.7±11.6	156.4±6.2	141±3.7	37.04±0.9	34.67±0.7
0.6	232.9±7.8	126.8±2.5	178±3.7	81.9±4.1	42.07±0.5	8.44±0.12
0.9	252.9±4.6	36.8±6.3	189±3.9	44±1.2	49.36±0.08	2.33±0.06

Values represent the mean ± SD (N=10)

barbarum by *A. tumefaciens*, confirmed by Southern hybridization. RT-PCR showed that *ATHK1* could be stably expressed in transgenic *L. barbarum*. No constitutive transcript accumulation in hybridization signals in Southern blotting and RT-PCR were detected in the WT, indicating that the gene is not endogenously present in the *L. barbarum* genome. The selected independent transgenic lines showed normal WT growth and morphology under normal conditions over a 6-month observation period.

All of our data indicated that the transgenic *L. barbarum* plants were more tolerant than WT to water deficit and salt stress. Transgenic plants showed normal growth even under extreme drought and high salt conditions, while WT plants became wrinkled and lost color, often dying following extreme stress. In addition, when transferred to standard water and soil conditions after a stress treatment, the transgenic lines showed faster recovery than WT plants. WT *L. barbarum* seedlings could not survive when the maximum neutral salt concentration exceeded 0.9% NaCl. However, neutral salt concentration lower than 1.5% NaCl could not influence the transgenic plant seedlings.

High water level maintenance in plants is an indication of high drought and salt tolerance and can be expressed as relative water content (Flower and Ludlow 1986). RWC is frequently used as screening tool for drought and salinity tolerance, for example in cereals and sunflower (Talamè et al. 2007; Rampino et al. 2006; Liu and Baird 2003; Teulat et al. 2003; Malatrasi et al. 2002). Maintenance of high RWC under dehydration and salinity stress can be attributed to *ATHK1* in transgenic *L. barbarum*. The water loss ratio of excised leaves was used as an indicator of osmotic resistance (Hao et al. 2004) and was substantially higher in transgenic plants than in WT plants. Water loss rate and RWC results both suggested the possibility of a respectively lower transpiration rate and an increased water uptake in the transgenic *L. barbarum* plants.

In general, osmotically resistant plants have better adaptability to drought and high salinity conditions than do less resistant plants due to reduced alteration of cell membrane permeability during osmotic adjustment in water deficit and high salinity environment. The cell membrane permeability is reflected by its conductance value, which is proportional to increased ion leakage ratio, a serious impairment in WT wheat cell membranes (Bajji et al. 2004). In our experiments, cellular membrane stability in the transgenic lines seemed to be unaffected by water deficit and high salinity conditions, whereas in WT plants, it is badly damaged. This result suggested an increased ability of the transgenic plants to counteract the damaging effects of salinity and drought stress on endogenous cell membranes, which allowed the transgenic plants to survive under the stress conditions imposed in this study.

Proline has also been reported to contribute to cellular membrane stability (Ashraf and Harris 2004; Hanson and Burnet 1994). Its accumulation is considered to enable proline to act as an important osmolyte (Behera et al. 2003), allowing osmotic adjustment under water deficit and salt stress conditions (Nanjo et al. 1999; Verslues and Bray

Table 2 Changes in protective enzymes activities of transgenic and WT *L. barbarum* L plants under drought stress

Unwatered (days)	Protective enzyme activity					
	SOD ($U \cdot g^{-1} \cdot h^{-1}$ FW)		POD ($U \cdot g \cdot g^{-1}$ FW)		CAT ($U \cdot g^{-1} \cdot min^{-1}$ FW)	
	Transgenic	WT (control)	Transgenic	WT (control)	Transgenic	WT (control)
0	179.3±7.1	176.9±5.8	149±5.2	142±5.8	24.27±0.6	24.21±0.2
15	209.3±5.7	72.7±1.6	176.4±6.9	41±5.7	37.04±0.9	18.43±0.5
30	216.9±5.4	22.8±2.5	178.9±7.3	11.9±3.7	42.07±0.5	2.56±0.49

Values represent the mean ± SD (N=10)

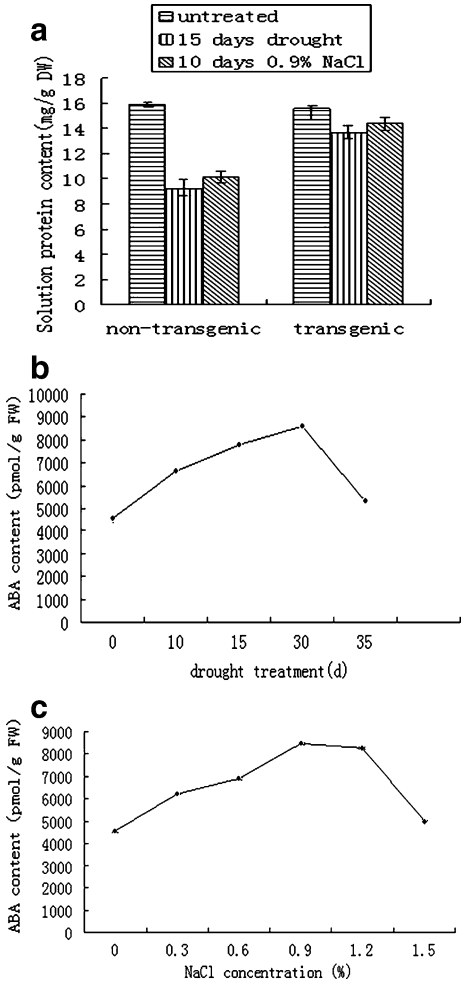

Fig. 6 Effects of drought and salinity stress on soluble protein content in leaf discs of *ATHK1* transgenic and WT *L. barbarum* plants (**a**). Changes in ABA content in transgenic *L. barbarum* under different days of drought treatment (**b**) and increasing NaCl concentrations (**c**). Data represent the means ± SD of three replicates

2004). In *Arabidopsis*, proline-deficient mutants are hypersensitive to osmotic stress (Brini et al. 2007). Our results confirmed again that increased endogenous proline levels in transgenic *L. barbarum* under salinity and drought treatments were associated with enhanced ability of plants to tolerate osmotic stress.

Chlorophyll changes are fundamental to understanding a plant's responses to the environment in which it resides (Schlemmer et al. 2005). The chlorophyll content increased in *Amaranthus tricolor* treated with 300 mM NaCl for 7 days (Wang and Nii 2000). Graminaceous chlorophyllic cells subjected to osmotic stress developed substantially higher amount of chlorophyll (García-Valenzuela et al. 2005). On the other hand, it has been reported that many different proteins are synthesized and accumulated in response to osmotic stress, including dehydrins, heat shock proteins, and detoxification proteins (Campalans et al. 1999; Smirnoff 1998). PEG treatment was found to decrease the soluble protein content in leaf discs of gerbera (Lai et al. 2007). Our results showed that after salt and drought treatments, the degradation of chlorophyll and soluble protein was much lower in leaves of transgenic *Lycium* plants than in WT plants.

ROS at high concentrations are highly cytotoxic, inducing DNA damage, lipid peroxidation, and protein degradation (Sun 1990), as well as membrane damage resulting in electrolyte leakage (Apse and Blumwald, 2002). Plants generate abundant ROS in form of O_2^- and H_2O_2 under salt and drought stresses (He et al. 2007; Luna et al. 2005). Lipid hydroperoxidation is another effective indicator of cellular oxidative damage and can be measured by changes in MDA content in leaves (Parvanova et al. 2004). In our study, there were no differences in O_2^- production rate, MDA, and H_2O_2 content in transgenic and non-transgenic plants before stresses were applied. However, a relatively strong increase of O_2^- production rate, MDA production, and H_2O_2 content was observed in WT plants, while only a slight increase was seen in transgenic plants expressing *ATHK1* under both drought and NaCl stress. The increased O_2^-, MDA, and H_2O_2 level further confirmed that WT *L. barbarum* produced excessive ROS under water deficit and salt stress. This slow increase in H_2O_2 and O^{2-} may have been responsible for the much stronger tolerance of transgenic lines treated under different concentrations of NaCl and water deficit.

Plants also possess systems to scavenge ROS to prevent the destructive oxidative reactions (Foyer et al. 1994). These consist of a number of antioxidant defense systems found in aerobic cells to counteract the damaging effects of ROS (Tsai et al. 2005; Lee et al. 2001; Orendi et al. 2001). The induction of ROS-scavenging enzymes such as SOD, POD, and CAT is the most common response to ROS synthesized under environmental stress (Lai et al. 2007). SOD and POD activities have been found to be enhanced in osmotically stressed leaf discs of rape plants compared to unstressed control plants (Aziz and Larher 1998). In our study, under normal conditions, enzyme activities of CAT, SOD, and POD remained unchanged in both transgenic and

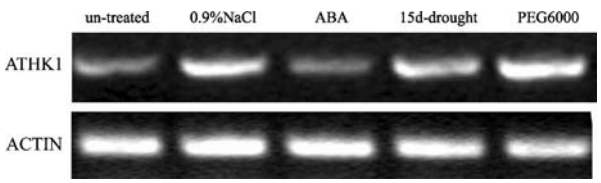

Fig. 7 Expression level of *ATHK1* in transgenic plants under various stress treatments. Two micrograms of RNA from 8-week-old plants that had been treated with 0.9% NaCl, 100 μM ABA, 15 days drought or PEG6000 were subjected to semiquantitative RT-PCR. ACTIN: RNA was used as an internal template control

WT lines. When subjected to drought and salt stresses, a rapid decrease in CAT, SOD, and POD activity was observed in WT *L. barbarum*, while the activities of SOD, CAT, and POD were greatly increased in transgenic plants by drought and high NaCl stresses. The transgenic lines in our study showed less accumulation of O_2^-, MDA, H_2O_2 and higher activity of SOD, CAT, and POD, which were believed to protect plants from salinity and water deficit injuries. These results suggest that expression of *ATHK1* may play a crucial role in preventing the over-accumulation of ROS by ROS-scavenging activities, thus protecting cell membrane integrity and enhancing salinity and drought tolerance in transgenic *L. barbarum* plants.

ATHK1 mRNA was expressed in transgenic *L. barbarum* and increased after drought, salt, and osmotic stress treatments, suggesting that it has a function in transgenic lines. *ATHK1* positively regulates many ABA-inducible genes and controls stress responses in *Arabidopsis* through both ABA-dependent and independent signaling pathways (Wohlbach et al. 2008; Tran et al. 2007a). It appears that dehydration triggers the production of ABA, which in turn induces the expression of different genes. Several genes that are induced by water stress are not responsive to exogenous ABA treatment (Shinozaki and Yamaguchi-Shinozaki 2007). Our findings suggest that ABA did not induce increased expression of *ATHK1* gene in transgenic lines, although endogenous ABA was accumulated rapidly under drought and salinity stress.

In this study, we observed that ATHK1 under CaMV 35S promoter may function as a plant osmosensor and play an important role in an osmotic stress signaling pathway in transgenic *L. barbarum*. The ATHK1 protein is likely involved in regulating the transcription of other related stress genes in transgenic *L. barbarum*, but the altered expression of a large number of stress-responsive genes involved in osmotic stress pathway still needs to be investigated. The regulatory role of ATHK1 in transgenic *L. barbarum* also needs further study.

Statistical evaluation of our data showed that all the transgenic plants performed better than did the non-transgenic plants under water deficit and high salinity conditions. This indicated that transgenic *L. barbarum* had a more enhanced salt and drought tolerance than did WT plants. To date, this is the first report on the expression of *ATHK1* in *L. barbarum* by means of an *A. tumefaciens*-mediated gene transfer system. The activation of several stress-inducible pathways and accumulation of several metabolites or antioxidants in transgenic plants may represent a new way to develop plants that are able to survive stress conditions through osmotic regulation. Our next task will be to assess the growth performance of these transgenic plants in the field when subjected to a combination of drought and salinity stresses.

Acknowledgements This work was financially supported by the grant received under the National Natural Science Foundation of China (No. 30771091), the Key grant Project of Chinese Ministry of Education (NO: 307024) and the Plan of Talent Scout of Lanzhou University (2004).

References

Aebi H, Wyss SR, Scherz B et al (1974) Heterogeneity of erythrocyte catalase II. Isolation and characterization of normal and variant erythrocyte catalase and their subunits. Eur J Biochem 48:137–145, doi:10.1111/j.1432-1033.1974.tb03751.x

Amtmann A, Bohnert HJ, Bressan RA (2005) Abiotic stress and plant genome evolution. Search for new models. Plant Physiol 138:127–130, doi:10.1104/pp.105.059972

Apse MP, Blumwald E (2002) Engineering salt tolerance in plants. Curr Opin Biotechnol 13:146–150, doi:10.1016/S0958-1669(02)00298-7

Arnon DI (1949) Copper enzymes in isolated chloroplasts in *Beta vulgaris*. Plant Physiol 24:1–15

Ashraf M, Harris PJC (2004) Potential biochemical indicators of salinity tolerance in plants. Plant Sci 166:3–16, doi:10.1016/j.plantsci.2003.10.024

Aziz A, Larher F (1998) Osmotic stress induced changes in lipid composition and peroxidation in leaf discs of *Brassica napus* L. J Plant Physiol 153:754–762

Bajji M, Kinet JM, Lutts S (2004) The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. Plant Growth Regul 36:61–70, doi:10.1023/A:1014732714549

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Crit Rev Plant Sci 24:23–58, doi:10.1080/07352680590910410

Bates LS, Waldren RP, Teeare ID (1973) Rapid determination of free Pro for water-stress studies. Plant Soil 39:205–207, doi:10.1007/BF00018060

Behera SK, Nayak L, Biswal B (2003) Senescing leaves possess potential for stress adaptation: the developing leaves acclimated to high light exhibit increased tolerance to osmotic stress during senescence. J Plant Physiol 160:125–131, doi:10.1078/0176-1617-00791

Boyer JS (1982) Plant productivity and environment. Science 218:443–448, doi:10.1126/science.218.4571.443

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254, doi:10.1016/0003-2697(76)90527-3

Bray EA (2004) Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. J Exp Bot 55:2331–2341

Brini F, Hanin M, Lumbreras V, Amara I, Khoudi H, Hassairi A, Pagès M, Masmoudi K (2007) Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in *Arabidopsis thaliana*. Plant Cell Rep 26:2017–2026, doi:10.1007/s00299-007-0412-x

Campalans A, Messeguer R, Goday A, Pagès M (1999) Plant responses to drought, from ABA signal transduction events to the action of the induced proteins. Plant Physiol Biochem 37:327–340, doi:10.1016/S0981-9428(99)80039-4

Chang RC, So KF (2007) Use of anti-aging herbal medicine, *Lycium barbarum*, against aging-associated diseases. What do we know so far? Cell Mol Neurobiol 28:643–652, doi:10.1007/s10571-007-9181-x

Committee of Chinese Pharmacopoeia, Ministry of Public Health of PR China (1990) Chinese pharmacopoeia, vol 1. Public Health Press and Chemical Industry Press, Beijing

Du GL, Song CZ, Zhang GL et al (2006) Expression of HIV capsid protein in transgenic *Lycium barbarum* L. and identification of expressed product. Chin J Biologicals 19:127–129 (in Chinese)

Elstner EF, Heupel A (1976) Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. Anal Biochem 70:616–620, doi:10.1016/0003-2697(76)90488-7

Flower DJ, Ludlow MM (1986) Contribution of osmotic adjustment to the dehydration tolerance of water-stressed pigeonpea [*Cajanus cajan* (L.) Millsp.] leaves. Plant Cell Environ 9:33–40

Foyer CH, Lelandais M, Kunert KJ (1994) Photooxidative stress in plants. Physiol Plant 92:696–717, doi:10.1111/j.1399-3054.1994.tb03042.x

García-Valenzuela X, Garcá-Moya E, Rascón-Cruz Q, Herrera-Estrella L, Aguado-Santacruz GA (2005) Chlorophyll accumulation is enhanced by osmotic stress in graminaceous chlorophyllic cells. J Plant Physiol 162:650–661, doi:10.1016/j.jplph.2004.09.015

Giannopolitis CN, Ries SK (1977) Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol 59:309–314

Hanson AD, Burnet M (1994) Evolution and metabolic engineering of osmoprotectant accumulation in higher plants. In: Cherry JH (ed) Cell biology: biochemical and cellular mechanism of stress tolerance in plants, NATO ASI Series H. Springer, Berlin, pp 291–302

Hao GP, Wu ZY, Chen MS et al (2004) ATHK1 gene regulates signal transduction of osmotic stress in *Arabidopsis thaliana*. J Plant Physiol Mol Biol 30:553–560 (in Chinese)

He Z, He C, Zhang Z et al (2007) Changes of antioxidative enzymes and cell membrane osmosis in tomato colonized by arbuscular mycorrhizae under NaCl stress. Colloids Surf B Biointerfaces 59:128–133, doi:10.1016/j.colsurfb.2007.04.023

Hu Z, Yang J, Guo GQ et al (2002) High efficiency transformation of *Lycium barbarum* and mediated by *Agrobacterium tumefaciens* and transgenic plant regeneration via somatic embryogenesis. Plant Cell Rep 21:233–237, doi:10.1007/s00299-002-0462-z

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. Annu Rev Plant Physiol Plant Mol Biol 47:377–403, doi:10.1146/annurev.arplant.47.1.377

Jenks MA, Joly RJ, Peters PJ et al (1994) Chemically induced cuticle mutation affecting epidermal conductance to water vapor and disease susceptibility in *Sorghum bicolor* (L.) Moench. Plant Physiol 105:1239–1245

Lai QX, Bao ZY, Zhu ZJ et al (2007) Effects of osmotic stress on antioxidant enzymes activities in leaf discs of PSAG12-IPT modified Gerbera. J Zhejiang Univ Sci B 8:458–464, doi:10.1631/jzus.2007.B0458

Lee DH, Kim YS, Lee CB (2001) The inductive responses of the antioxidant enzymes by salt stress in the rice (*Oryza sativa* L.). J Plant Physiol 158:737–745, doi:10.1078/0176-1617-00174

Lin HM, Zhang YF, Jia HX et al (2007) Physiological and biochemical characteristics of leaf blade on different age branches of *Lycium barbarum* L. under salt stress. Chin J Eco Agric 15:112–114

Liu X, Baird WV (2003) Differential expression of genes regulated in response to drought or salinity stress in sunflower. Crop Sci 43:678–687

Liu H, Guo GQ, He YK et al (2007) Visualization on intercellular movement of chromatin in intact living anthers of transgenic tobacco expressing histone 2B-CFP fusion protein. Caryologia 60:1–20, doi:10.1159/000096925

Luna CM, Pastori GM, Driscoll S et al (2005) Drought controls on H_2O_2 accumulation, catalase (CAT) activity and CAT gene expression in wheat. J Exp Bot 56:417–423, doi:10.1093/jxb/eri039

Ma S, Bohnert HJ (2007) Integration of *Arabidopsis thaliana* stress-related transcript profiles, promoter structures, and cell-specific expression. Genome Biol 8:R49, doi:10.1186/gb-2007-8-4-r49

Ma S, Gong Q, Bohnert HJ (2006) Dissecting salt stress pathways. J Exp Bot 57:1097–1107, doi:10.1093/jxb/erj098

Maggio A, Zhu JK, Hasegawa PM et al (2006) Osmogenetics: Aristotle to Arabidopsis. Plant Cell 18:1542–1557, doi:10.1105/tpc.105.040501

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 444:139–158, doi:10.1016/j.abb.2005.10.018

Malatrasi M, Close TJ, Marmiroli N (2002) Identification and mapping of a putative stress response regulator gene in barley. Plant Mol Biol 50:143–152, doi:10.1023/A:1016051332488

Nanjo T, Kobayashi M, Yoshiba Y et al (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Lett 461:205–210, doi:10.1016/S0014-5793(99)01451-9

Orendi G, Zimmermann P, Baar C et al (2001) Loss of stress-induced expression of catalase 3 during leaf senescence in *Arabidopsis thaliana* is restricted to oxidative stress. Plant Sci 161:301–314, doi:10.1016/S0168-9452(01)00409-5

Parvanova D, Ivanov S, Konstantinova T et al (2004) Transgenic tobacco plants accumulating osmolytes show reduced oxidative damage under freezing stress. Plant Physiol Biochem 42:57–63, doi:10.1016/j.plaphy.2003.10.007

Rampino P, Pataleo S, Gerardi C et al (2006) Drought stress response in wheat: physiological and molecular analysis of resistant and sensitive genotypes. Plant Cell Environ 29:2143–2152, doi:10.1111/j.1365-3040.2006.01588.x

Rizhsky L, Hallak-Herr E, Van Breusegem F et al (2002) Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. Plant J 32:329–342, doi:10.1046/j.1365-313X.2002.01427.x

RoyChoudhury A, Roy C, Sengupta DN (2007) Transgenic tobacco plants overexpressing the heterologous lea gene Rab16A from rice during high salt and water deficit display enhanced tolerance to salinity stress. Plant Cell Rep 26:1839–1859, doi:10.1007/s00299-007-0371-2

Sambrook J, Russell DW (2000) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Press, New York

Schlemmer MR, Francis DD, Shanahan JF et al (2005) Remotely measuring chlorophyll content in corn leaves with differing nitrogen levels and relative water content. Agron J 97:106–112

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot 58:221–227, doi:10.1093/jxb/erl164

Smirnoff N (1998) Plant resistance to environmental stress. Curr Opin Biotechnol 9:214–219, doi:10.1016/S0958-1669(98)80118-3

Sun Y (1990) Free radicals, antioxidant enzymes, and carcinogenesis. Free Radic Biol Med 8:583–599, doi:10.1016/0891-5849(90)90156-D

Talamè V, Ozturk NZ, Bohnert HJ et al (2007) The dynamics of water loss affects the expression of drought-related genes in barley. J Exp Bot 58:229–240, doi:10.1093/jxb/erl163

Tayal D, Srivastava PS, Bansal KC (2004) Transgenic crops for abiotic stress tolerance. In: Srivastava PS, Narula A, Srivastava S (eds) Plant biotechnology and molecular markers. Springer, The Netherlands, pp 346–365

Teulat B, Zoumarou-Wallis N, Rotter B et al (2003) QTL for relative water content in field-grown barley and their stability across Mediterranean environments. Theor Appl Genet 108:181–188, doi:10.1007/s00122-003-1417-7

Tran LS, Nakashima K, Shinozaki K et al (2007a) Plant gene networks in osmotic stress response: from genes to regulatory networks. Methods Enzymol 428:109–128, doi:10.1016/S0076-6879(07)28006-1

Tran LS, Urao T, Qin F et al (2007b) Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to

abscisic acid, drought, and salt stress in Arabidopsis. Proc Natl Acad Sci USA 104:20623–20628, doi:10.1073/pnas.0706547105

Tsai YC, Hong CY, Liu LF et al (2005) Expression of ascorbate peroxidase and glutathione reductase in roots of rice seedlings in response to NaCl and H_2O_2. J Plant Physiol 162:291–299, doi:10.1016/j.jplph.2004.06.004

Urao T, Miyata S, Yamaguchi-Shinozaki K et al (2000) Possible His to Asp phosphorelay signaling in an Arabidopsis two-component system. FEBS Lett 478:227–232, doi:10.1016/S0014-5793(00)01860-3

Urao T, Yamaguchi-Shinozaki K, Shinozaki K (2001) Plant histidine kinases: an emerging picture of two-component signal transduction in hormone and environmental responses. Sci STKE 109:RE18

Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain treated bean plants. Protective role of exogenous polyamines. Plant Sci 151:59–66, doi:10.1016/S0168-9452(99)00197-1

Verslues PE, Bray EA (2004) LWR1 and LWR2 are required for osmoregulation and osmotic adjustment in Arabidopsis. Plant Physiol 136:2831–2842, doi:10.1104/pp.104.045856

Walker-Simmons M (2005) ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. Plant Physiol 84:61–66

Wang Y, Nii N (2000) Changes in chlorophyll, ribulose bisphosphate carboxylase-oxygenase, glycine betaine content, photosynthesis and transpiration in *Amaranthus tricolor* leaves during salt stress. J Hortic Sci Biotechnol 75:623–627

Weigel D, Glazebrook J (2004) Arabidopsis: a laboratory manual, English gravure. Chemical Industry Press, China, pp 172–173

Wohlbach DJ, Quirino BF, Sussman MR (2008) Analysis of the Arabidopsis histidine kinase ATHK1 reveals a connection between vegetative osmotic stress sensing and seed maturation. The Plant Cell 20:1101–1117

Xie C, Xu LZ, Li XM et al (2001) Studies on chemical constituents in fruit of *Lycium barbarum* L. J Chin Materia Med 26:323–324 (in Chinese)

Xiong L, Zhu JK (2002) Molecular and genetic aspects of plant responses to osmotic stress. Plant Cell Environ 25:131–139, doi:10.1046/j.1365-3040.2002.00782.x

Xu YH, Xu Y, An WT (2000) The progress in studies on anti-tumor pharmacodynamics of *Lycium barbarum*. Lishizhen Med Mater Med Res 11:946–947 (in Chinese)

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781–803, doi:10.1146/annurev.arplant.57.032905.105444

Yang L, Tang R, Zhu J et al (2008) Enhancement of stress tolerance in transgenic tobacco plants constitutively expressing AtIpk2beta, an inositol polyphosphate 6-/3-kinase from *Arabidopsis thaliana*. Plant Mol Biol 66:329–343, doi:10.1007/s11103-007-9267-3

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273, doi:10.1146/annurev.arplant.53.091401.143329