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ORIGINAL PAPER

A Transient Transformation System for the Functional Characterization of Genes Involved in Stress Response

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Abstract In this study, we developed a simple and efficient transient transformation system, which can be used in homologous expression or reverse genetic study of the plants. A system for characterizing gene function in response to stress tolerance was also developed based on this transformation method. The overexpression and RNAi-silencing of a *bZIP* gene from *Tamarix hispida*, *ThbZIP1*, were performed in *T. hispida* using this transformation method. Real-time PCR showed that the expression of *ThbZIP1* was highly up- and down-regulated in the plants with overexpression and RNAi-silenced expression of *ThbZIP1*, respectively, when compared with control plants (transiently transformed with empty pROK2). A physiological study showed that *ThbZIP1* can enhance the activities of both peroxidase (POD) and superoxide dismutase (SOD), and decrease electrolyte leakage rate and levels of reactive oxygen species (ROS) and malondialdehyde (MDA) under salt stress conditions. Furthermore, *ThbZIP1* is found to mediate stress tolerance by regulating the expression of *SOD* and *POD* genes. These results suggested that this transient transformation system is an effective method for determining the function of a gene in response to abiotic stress in plants.

Keywords bZIP transcription factor · Abiotic stress · Transient transformation system · Homologous overexpression · RNAi-silenced · *Tamarix hispida*

Introduction

Genetic transformation is a powerful method for studying the functions and regulatory mechanisms of genes involved in various physiological and biochemical processes. *Agrobacterium tumefaciens*-mediated transformation is considered the most effective genetic transformation method and has been widely used in plant transformation. However, generation of stably transformed transgenic plants is time consuming, and it is also impossible for many plant species that lack genetic transformation systems. Transient gene expression systems complement stable transformation and provide novel experimental tools for physiological and localization studies, and for generating gene products (Kirienko et al. 2012; Chen et al. 2010). In comparison with a stable transformation, transient transformation has several advantages: gene expression can be analyzed very shortly after DNA delivery; it does not interfere with the stability of the host genome (Lu et al. 2013); gene expression is not influenced by positional effects; and it does not require regeneration of a transformed cell in order to analyze the transformation. Moreover, transient transformation systems can dramatically enhance the speed of research, as many constructs can be analyzed in parallel within a short time frame (Li et al. 2009b). These advantages make these systems powerful methods in research, especially in plants that are recalcitrant to regeneration (Kapila et al. 1997), and they are widely used for studying the function or transient activities of genes (Koroleva et al. 2005; Yoo et al. 2007).

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In order to study a gene's function in plant cells, various transient expression systems have been developed. These methods include particle bombardment (Klein et al. 1988), the polyethylene glycol (PEG)-mediated protoplast transformation (Yoo et al. 2007), *A. tumefaciens* vacuum infiltration (Simmons et al. 2009; Takata and Eriksson 2012), and agroinfiltration methods (Kapila et al. 1997; Yang et al. 2000; Goodin et al. 2002). Most of these transient transformation methods are based on *Agrobacterium* infection, and *Agrobacterium*-mediated transformation techniques have been confirmed to be powerful in gene transfer. Among these transformation methods, leaf infiltration is the most efficient and commonly used method for transient gene expression. However, this method is only appropriate for a few plant species, which are broadleaf plants that are easily injectable, such as *Arabidopsis* (Wroblewski et al. 2005), radish, pea, lupine, and flax (Van der Hoorn et al. 2000), tobacco (Sheludko et al. 2006), lettuce, tomato, and switchgrass (VanderGheynst et al. 2008), but it cannot be used in plants with needle-shaped leaves or leaf blades. Particle bombardment systems can be used on various plant species, but it requires a particle bombardment device, which is costly. Additionally, the procedure of particle bombardment is relatively complex. Furthermore, particle bombardment is usually used in tissue transformation and is not very suitable for whole-plant transformation.

To date, most plant species do not have a regeneration system and a genetic transformation system, which is a major limiting factor in functionally characterizing the genes from these plant species, and only some model plants such as *Arabidopsis*, rice, tomato, wheat, and some poplar species that have efficient genetic transformation systems have been intensively studied at the molecular level. Therefore, development of efficient genetic transformation systems for a plant species is quite important for the characterization of gene function and molecular biological studies in that plant. Establishment of regeneration and transformation systems is difficult for many plant species, especially for woody plants. Therefore, developing simple and efficient transient expression systems is a complement for stable transformation methods and may be useful for molecular physiological studies.

Previously, we established a transient transformation method (Zheng et al. 2012). In this paper, we optimized this transient transformation method and further developed a system for characterizing gene function in response to stress tolerance based on this method. The function of a *bZIP* gene from *Tamarix hispida* in response to salt stress was analyzed using this method, and the results confirmed that this method is reliable and powerful in determining the function of a gene in response to abiotic stresses.

Materials and Methods

Plant Materials

The seedlings of *Tamarix hispida* were cultured in 1/2 MS containing 2.5 % (w/v) sucrose and 0.6 % (w/v) agar. Five-week-old *T. hispida* seedlings (grown on 1/2 MS medium) were employed as host plants for transformation. The plantlets of birch, poplar, cork, willow, and aralia grown in tubes were used as host plants for a transient transformation assay. *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA1301 was used in the transformation studies.

Plasmid Construction

For the overexpression of *ThbZIP1* (GenBank number: FJ752700), the ORF of *ThbZIP1* was cloned into pROK2 under control of the 35S promoter (35S:bZIP). To silence the expression of *ThbZIP1*, an inverted repeat cDNA fragment of *ThbZIP1* was inserted into the binary plasmid pFGC5941 (pFGC: *bZIP*). All the primers used are shown in Supporting information Table S1.

Transformation

Single colonies of *A. tumefaciens* strain EHA105 harboring 35S:bZIP, pFGC:bZIP, or empty pROK2 were each grown in LB medium (containing 50 mg/L kanamycin and 50 mg/L rifampicin) at 28 °C with shaking. After overnight incubation, 0.5 ml of culture was transferred to 25 ml of fresh liquid media and incubated at 28 °C with shaking. Cells were harvested by centrifugation at 3,000 rpm for 10 min when the culture density reached an OD600 of 0.6, and the centrifuged cells were adjusted to an OD600 of 0.9 with 1/2 MS solution [150 μM acetosyringone, 2.5 % (w/v) sucrose, and 0.01 % (w/v) Tween20, pH 5.8] as the transformation solution for the transformation study. Before transformation, the plant seedlings were soaked in 1/2 MS solution [25 % (w/v) sucrose, pH 5.8] for 2 h for hyperosmotic pretreatment. For transient transformation, after hyperosmotic treatment, plant seedlings were soaked in the transformation solution and incubated at 25 °C with shaking at 120 rpm for 6 h. The seedlings were then washed twice with distilled water. The plants were grown vertically on 1/2 MS agar medium [150 μM acetosyringone, 2.5 % (w/v) sucrose, pH 5.8]. If obvious strain colonies occurred on the medium around the plantlets, the plants were moved to another location on the same plate. This is the basic transformation procedure for all the experiments performed here.

Biochemical Staining

After culture on agar medium for 66 h (72 h of transformation, in fact, including 6 h of being soaked in liquid medium), the plantlets were harvested for biochemical staining. *T. hispida* plants overexpressing *ThbZIP1*, RNAi-silenced plants and control plants subjected to the 125 mM NaCl treatment were infiltrated with 3, 30-diaminobenzidine (DAB) solution or nitroblue tetrazolium (NBT) following the procedures described by Zhang et al. (2011). Cell death was examined by Evans blue staining as described by Kim et al. (2003). In each biochemical staining experiment, at least five plantlets were used.

Physiological Measurements

After being cultured on 1/2 MS agar medium for 42 h (48 h of transformation, including 6 h of being soaked in liquid medium), the plantlets were moved to plates containing medium with 0.2 M NaCl for stress induction for 48 h. Then, the plantlets were harvested for physiological analyses. SOD, POD, and MDA measurements were conducted as in Wang et al. (2010). H_2O_2 levels were measured according to Dal Santo et al. (2012) with little modification. For electrolyte leakage measurement, the leaves of each sample were rinsed with deionized water, round sections were cut from the leaves, and put into 30 ml of deionized water under vacuum for 15 min. The electrical conductivity was measured and marked as S1. Then, the leaves were heated at 90 °C for 20 min and cooled at room temperature. Electrical conductivity was measured, and was marked as S2. The formula for the calculation of electrolyte leakage was: (S1/S2)×100 %. Biological replicates were performed in triplicate.

Real-Time RT-PCR Analysis

For real-time RT-PCR analysis, the plantlets (whole) were harvested at various time points. First, after being cultured on 1/2 MS agar medium for 42 h (after transformation for 48 h in fact, including 6 h of soaked in liquid medium), some plantlets were harvested for real-time RT-PCR analysis. Second, the plantlets were moved to plates containing medium with 0.2 M NaCl and were harvested after stress induction for 24 and 48 h (after transformation for 72 and 96 h, in fact, including 6 h of soaked in liquid medium). Total RNA was isolated from each sample using a CTAB method (Chang et al. 1993), and was treated with DNaseI to remove any DNA residue. About 0.5 μg of total RNA was reverse-transcribed into cDNA with oligo (deoxythymidine) primers using PrimeScript™ RT reagent Kit (TaKaRa, China) in a reaction volume of 10 μL, and the procedure was followed by the protocol. The synthesized cDNAs were diluted to 100 μL with sterile water and used as the template for real-time PCR. The *α-tubulin* (GenBank number: EH050602), *β-tubulin* (GenBank number: EH056816) and *β-actin* (GenBank number: EG971352) genes were used as internal references. The primers used in PCR are shown in Supporting information Table S1. Real-time RT-PCR was performed with an Opticon 2 System (Bio-Rad, Hercules, CA, USA). The reaction mixture contained 10 μL of SYBR Green Real-time PCR Master Mix (Toyobo), 0.5 μM each of forward and reverse primers, and 2 μL of cDNA template (equivalent to 100 ng of total RNA) in a total volume of 20 μL. Amplification was performed with the following cycling parameters: 94 °C for 30 s followed by 45 cycles at 94 °C for 12 s, 60 °C for 30 s, 72 °C for 40 s, and 1 s at 82 °C for plate reading. A melting curve was generated for each sample at the end of each run to assess the purity of the amplified products. Three biological replicates were performed, and expression levels were calculated from the cycle threshold according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Study of the Efficiency of the Transient Transformation System in Different Plant Species

A single colony of *A. tumefaciens* strain EHA105 harboring pCAMBIA1301 was grown in LB medium at 28 °C. Cells were harvested by centrifugation at 3,000 rpm for 10 min when the culture density reached an OD600 of 0.6, and the centrifuged cells were adjusted to an OD600 of 0.9 with 1/2 MS solution [150 μM acetosyringone, 2.5 % (w/v) sucrose, 0.01 % (w/v) Tween20, pH 5.8] as the transformation solution. Plantlets of *T. hispida*, birch (*Betula platyphylla*), willow (*Salix matsudana*), aralia (*Aralia mandshurica*), tobacco (*Nicotiana tabacum* Linn.), and *Arabidopsis thaliana* (L.) were soaked in 1/2 MS solution [25 % (w/v)] sucrose, pH 5.8) for 2 h for hyperosmotic pretreatment. Then, the plantlets were soaked in transformation solution (containing *Agrobacterium* EHA105 harboring pCAMBIA1301 at an OD600 of 0.9) at 25 °C for 5 h, washed twice, grown vertically on 1/2 MS agar medium [150 μM acetosyringone, 2.5 % (w/v) sucrose, pH 5.8], and cultured for 42 h. The GUS staining analyses were performed as described by Jefferson (1989).

Statistical Analysis

Data analyses were carried out using SPSS 16.0 (SPSS,Chicago, Il, USA) software. For all the data analyses, the level of significance was set at $P<0.05$. Sample variability is given as the standard deviation (SD).

Results

Generation of the Transiently Overexpressed and RNAi-silenced *ThbZIP1 T. hispida* Plants

In this study, a transient transformation system was developed, and transgenic *T. hispida* plants were generated. To investigate the expression of the transformed gene in the control (transformed with empty pROK2), overexpressing (transformed with 35S: bZIP), and RNAi-silenced (transformed with pFGC: bZIP) plants, real-time RT-PCR was performed. The expression level of *ThbZIP1* in control plants after being transformed for 48 h was used as the calibrator (designated as 0 after log2 transformed). The results showed that, compared with control plants, the expression of *ThbZIP1* greatly increased in the overexpressing *T. hispida*, especially after being transformed for 96 h. Meanwhile, the expression of *ThbZIP1* significantly decreased in the RNAi-silenced plants (Fig. 1). These results indicated that this transient transformation system is efficient for the transformation of genes.

Stress Tolerance Analysis of the *ThbZIP1* Transformed *T. hispida* Plants

After confirming that the transient transformation system works well in *T. hispida* plants and that the target genes were significantly altered in transgenic plants, we further determined whether this system can be used for characterizing the function of a gene in stress tolerance. Biochemical staining was performed first, and the plants were stained by DAB, NBT, and Evans Blue after transformation for 72 h. Two prominent ROS species, O^{2-} and H_2O_2, can be stained by

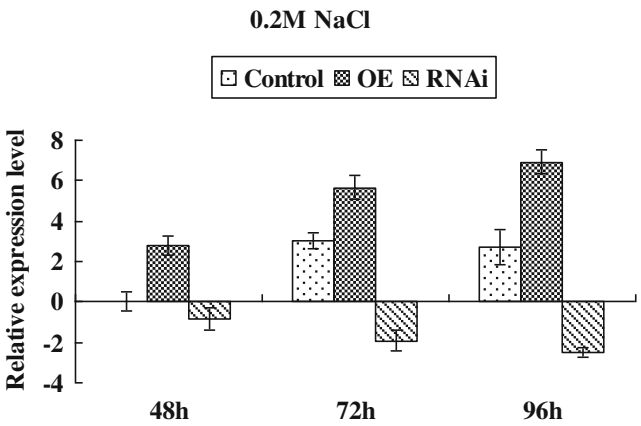

Fig. 1 Analyses of the expression of *ThbZIP1* in the control, *ThbZIP1*-overexpressing, and RNAi-silenced *ThbZIP1* plants. *Control T. hispida* plants transformed with empty pROK2; *OE T. hispida* plants overexpression of *ThbZIP1*; *RNAi ThbZIP1* RNAi-silenced *T. hispida* plants. The expression of *ThbZIP1* was determined after transformation for 48, 72, and 96 h. The expression level of *ThbZIP1* in control plants after transformation for 48 h was used as the calibrator (designated as 0 after log2 transformed). *Error bars* SD for each experiment

NBT and DAB in situ, respectively. Both DAB and NBT staining showed that overexpression of *ThbZIP1* can obviously reduce ROS levels compared with the control and RNAi-silenced *ThbZIP1* plants, indicating that overexpression of *ThbZIP1* can decrease ROS accumulation under salt stress (Fig. 2a, b). Evans Blue staining indicated that there is decreased cell death in transgenic plants overexpressing *ThbZIP1* under salt stress conditions compared with control and RNAi plants (Fig. 2c). Consistent with DAB staining, H_2O_2 content measurement also showed that there was no obvious difference in H_2O_2 content between transgenic and control plants under normal growth condition. However, under salt stress condition, H_2O_2 content in the *ThbZIP1* RNAi-silenced plants was highest, followed by control plants, while plants overexpressing *ThbZIP1* had the lowest H_2O_2 content (Fig. 2d). An electrolyte leakage assay showed that the electrolyte leakage rate is lowest in plants overexpressing *ThbZIP1*, followed by control plants, and then in *ThbZIP1* silenced plants (Fig. 2e). Taken together, we concluded that the *T. hispida* plants overexpressing *ThbZIP1* display decreased ROS accumulation and cell death under salt stress conditions, suggesting that *ThbZIP1* plays a positive role in stress tolerance regulation.

Previously, we reported that overexpression of *ThbZIP1* in tobacco plants under salt stress can improve stress tolerance of plants by enhancing SOD and POD activity and decreasing MDA content (Wang et al. 2010). In the present study, we further studied these physiological changes using a homologous expression system. The results showed that, after transformation for 48 h, there are no significant differences in POD and SOD activity among the control, overexpressing, and RNAi-silenced *ThbZIP1* plants (Fig. 3a, b). However, transgenic plants overexpressing *ThbZIP1* showed highly increased SOD and POD activity, followed by control plants, and then the RNAi-silenced plants (Fig. 3). These results are consistent with the results obtained from *ThbZIP1*-transformed tobacco, indicating that this transient transformation system is an accurate and reliable tolerance analysis. In addition, SOD and POD activity displays a linear relationship with the expression of *ThbZIP1*, suggesting that *ThbZIP1* can regulate SOD and POD activity (Figs. 1, 3a, b). Moreover, MDA content analysis showed that the plants overexpressing *ThbZIP1* had the lowest MDA content, and RNAi-silenced *ThbZIP1* plants had the highest MDA content (Fig. 3c), indicating that the expression level of *ThbZIP1* is inversely related to MDA accumulation.

Analysis of the Expression of the *PODs* and *SODs* in Transgenic Plants

The fact that the expression of *ThbZIP1* is linearly correlated with SOD and POD activity suggested that *ThbZIP1* may regulate the expression of the *SOD* and *POD* genes.

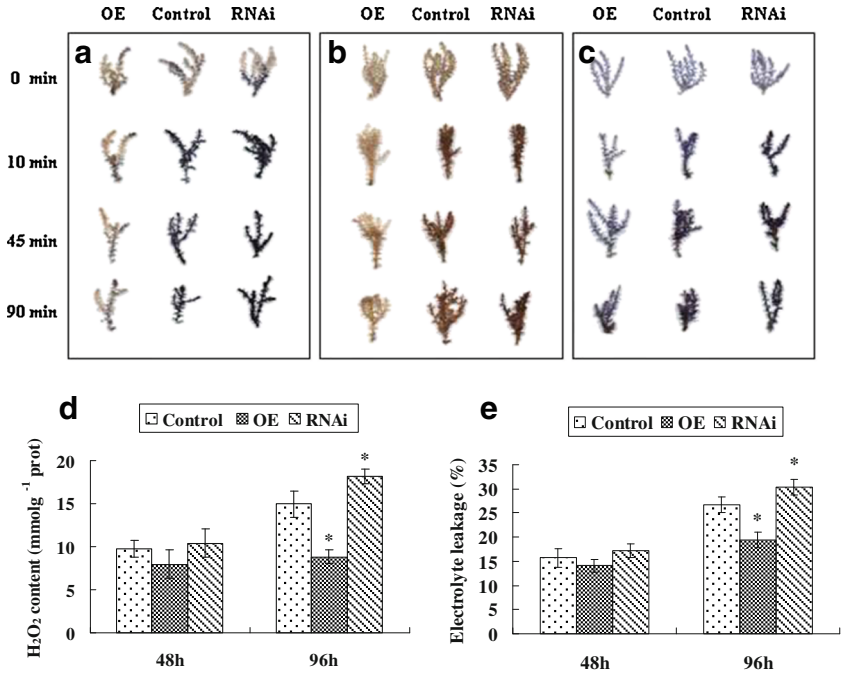

Fig. 2 Histochemical staining and related physiological changes analyses of transgenic *T. hispida* plants. **a**, **b** The plants were stained with NBT (**a**) and DAB (**b**) to reveal the accumulation of O^2- and H_2O_2, respectively. **c** Analysis of cell death by Evans Blue staining. After transformation for 72 h, the plants treated with 125 mM NaCl for 0, 10, 45, and 90 min, and were used for histochemical staining analysis. **d** Analysis of H_2O_2 level in the control, *ThbZIP1*-overexpressing, and RNAi-silenced *ThbZIP1* plants. **e** analyses of electrolyte leakage rate. *48 h* After transformation for 48 h without stress, the plantlets were harvested for analyses; *96 h* after transformation for 48 h, the plants were treated with 0.2 M NaCl for 48 h, and were harvested for analyses. *Control T. hispida* transformed with empty pROK2, *OE T. hispida* plants overexpressing *ThbZIP1*, *RNAi T. hispida* plants with RNAi-silenced *ThbZIP1*

Therefore, we further measured the expression of *SODs* and *PODs* using real-time RT-PCR. Three *SOD* (*ThSOD1*, *ThSOD2* and *ThSOD3*, with their GenBank number of KF756930, KF756931 and KF756932) and *POD* (*ThPOD1*, *ThPOD2* and *ThPOD3*, with their GenBank number of KF756934, KF756935 and KF756936) genes were cloned from *T. hispida* (Gao et al. 2010; Li et al. 2009a) and were further studied in the transiently transformed *T. hispida* plants. The results revealed that the expression levels of all the studied *SOD* and *POD* genes were enhanced in *ThbZIP1*-overexpressing plants compared with in control plants or in the *ThbZIP1*-silenced plants. Furthermore, 3 *POD* and 2 *SOD* genes showed significantly decreased expression in RNAi-silenced *ThbZIP1* plants compared with in control plants (Fig. 4). These results indicated that *ThbZIP1* can regulate expression of the *SOD* and *POD* genes, and the enhanced expression of the *SOD* and *POD* genes can improve *SOD* and *POD* activities, leading to enhanced stress tolerance. Taken together, these results showed that *ThbZIP1* can mediate stress tolerance by regulation of ROS scavenging systems.

The Transformation Method Works well in Different Plant Species

To explore whether this transient expression system can work for plants other than *T. hispida*, six additional plant species, including wood and grass plants (*T. hispida*, birch, willow, aralia, tobacco, and *Arabidopsis thaliana*), were further transformed with pCAMBIA1301 using this system. GUS staining showed that the *GUS* gene was expressed well in all of these plants (Fig. 5), demonstrating that this transformation system can work well in these plants.

Discussion

In the present study, we developed a transient expression system and a quick way to determine the stress tolerance of a gene based on this transient transformation. Real-time RT-PCR showed that *ThbZIP1* was highly expressed in plants transiently transformed with 35S: *bZIP* and highly inhibited in plants transiently transformed with pFGC: *bZIP* (RNAi-silenced *ThbZIP1*), which indicated that this transformation system is efficient for gene transformation, resulting in the overexpression or silencing of *ThbZIP1*. Previously, we overexpressed *ThbZIP1* in tobacco plants, and the transformed plants showed improved salt tolerance combined with improved SOD and POD activity and decreased MDA level (Wang et al. 2010). Consistent with our previous work, in this study, compared with control plants (transformed with empty pROK2), the transgenic *Tamarix* plants overexpressing *ThbZIP1* showed improved salt tolerance (Figs. 2, 3).

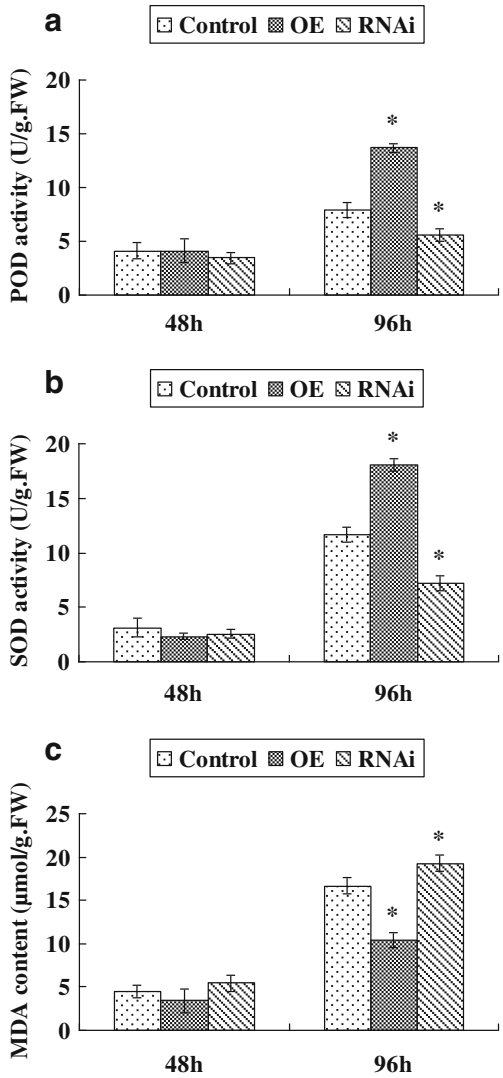

Fig. 3 Assay of POD and SOD activity and MDA content in *ThbZIP1* transgenic plants. **a**, **b** POD and SOD activity; **c** MDA content measurements. *48 h* After transformation for 48 h without stress, the plantlets were harvested for analyses; *96 h* after transformation for 48 h, the plants were treated with 0.2 M NaCl for 48 h, and were harvested for analyses. *Control T. hispida* transformed with empty pROK2, *OE T. hispida* plants overexpressing *ThbZIP1*, *RNAi T. hispida* plants with RNAi-silenced *ThbZIP1*

Meanwhile, the RNAi-silenced *ThbZIP1* plants displayed decreased stress tolerance (Figs. 2, 3). Additionally, SOD and POD activities were enhanced in the *ThbZIP1*-overexpressing plants, followed by Control plants, and then the RNAi-silenced *ThbZIP1* plants (Fig. 3). Furthermore, several *SOD* and *POD* genes were up-regulated in *ThbZIP1*-overexpressing plants and down-regulated in the RNAi-silenced plants (Fig. 4), indicating that *ThbZIP1* is involved in regulating the expression of the *SOD* and *POD* genes. These results clearly showed that the expression level of *ThbZIP1* is positively correlated with stress tolerance and the activities of SOD and POD. Taken together, these results indicated that this transient expression system is effective for the overexpression and RNAi silencing of genes. Therefore, it is a reliable method for the functional characterization of genes involved in stress tolerance.

Transient transformation systems are powerful tools for analyzing the function of genes and the generation of gene products (Chen et al. 2010). Therefore, developing an efficient transient transformation system for plants is important in plant biological studies. Some transformation methods, such as vacuum infiltration and agroinfiltration, need to get *Agrobacterium* cells into the plant tissues with an injection or vacuum. After the cells get into plant tissue, they can transfer the T-DNA into plant cells, resulting in the transient expression of the genes. In this study, we found that *Agrobacterium* cells can get into all the tissues of a plant when the plant is soaked in a liquid medium containing *Agrobacterium* cells for a certain period of time. Based on this, we developed a transient transformation method. This method is simple and easy, does not require the process of injection, a vacuum tool, or transformation devices, such as particle bombardment. In addition, this method has no limitations on the tissues of transformed plants, and tissues or whole seedlings can be transformed with a high efficiency of expression. It can transform genes by soaking plants in a liquid medium containing *A. tumefaciens* with an infiltration substance such as Tween-20 or silwet. However, this transient transformation method also has some limitations. For instance, it only transforms plants or tissues as a whole, and cannot be used in the transformation of parts of plant or tissues (such as leaf), or for performing more than one kind of transformation in a same leaf as in agroinfiltration. Also, the plants used in this transformation method should be sterile.

Reverse genetic methods, such as RNAi and antisense RNA, are an excellent strategy for reverse genetics. Additionally, RNAi-based silencing is found to be a powerful tool to silence the expression of genes and study their loss-of-function phenotype, especially for the genes whose mutant alleles are not available. RNAi has been used to generate a wide variety of loss-of-function phenotypes (Kuttenkeuler and Boutros 2004). A homologous expression system is important in the functional characterization of a gene, especially in reverse genetic studies. However, there are approximately 250,000 species of higher plants in the world (Phillipson 2001), and most of them, especially woody plants, do not have a tissue culture regeneration system. Because no transformation system is available, it is impossible to characterize the function of a gene by using homologous expression, RNAi, and antisense RNA methods. This is one of the major problems in performing molecular biology studies in these plants. Therefore, the development of a transient transformation system for the plants that do not have stable transformation may solve these problems. In the present study, we developed a transient transformation method which provides a possible way to perform homologous expression and reverse

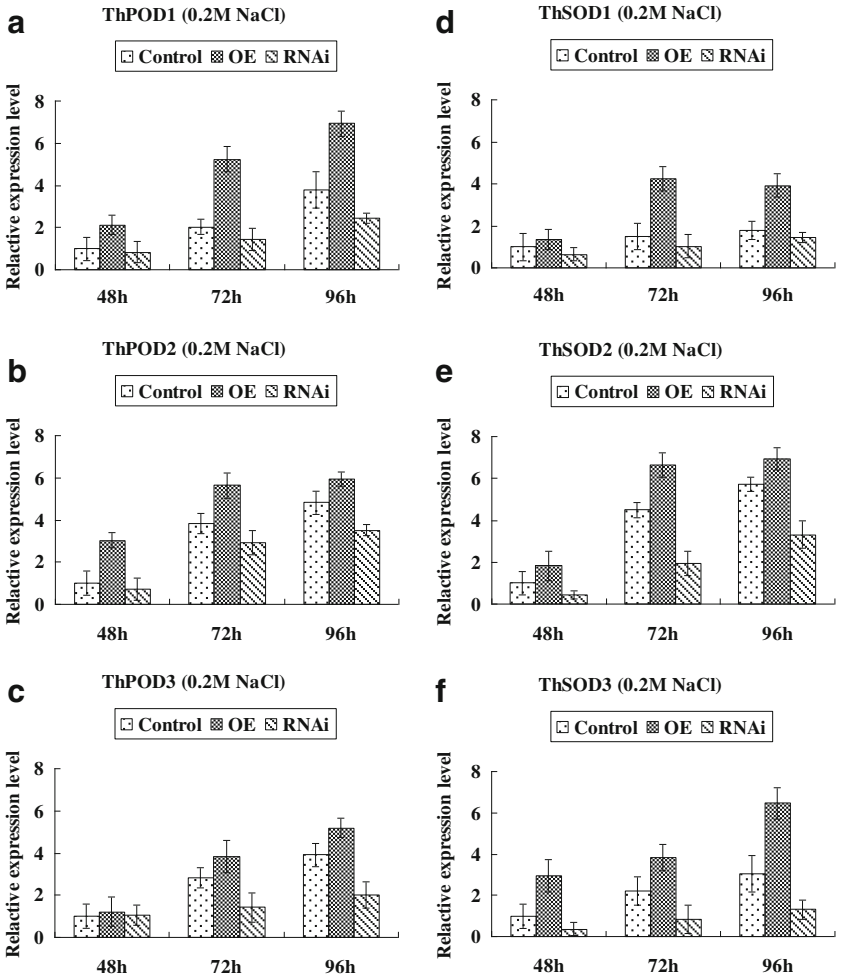

Fig. 4 Analysis of the expression of *SOD* and *POD* genes in the control, *ThbZIP1*-overexpressing, and RNAi-silenced *ThbZIP1* plants. Three *POD* and *SOD* genes from *T. hispida* were analyzed in control, *ThbZIP1*-overexpressing, and RNAi-silenced *ThbZIP1* plants. After transformation for 48 h, these plants were treated with 0.2 M NaCl for 0 (*48 h*), 24 (*72 h*) and 48 h (*96 h*), and gene expression was studied. **a**–**c** The expression of *ThPOD1* (GenBank number:KF756934), *ThPOD2* (GenBank number:KF756935), or *ThPOD3* (GenBank number:KF756936). **d**–**f** The expression of *ThSOD1* (GenBank number:KF756930), *ThSOD2* (GenBank number:KF756931), or *ThSOD3* (GenBank number:KF756932)

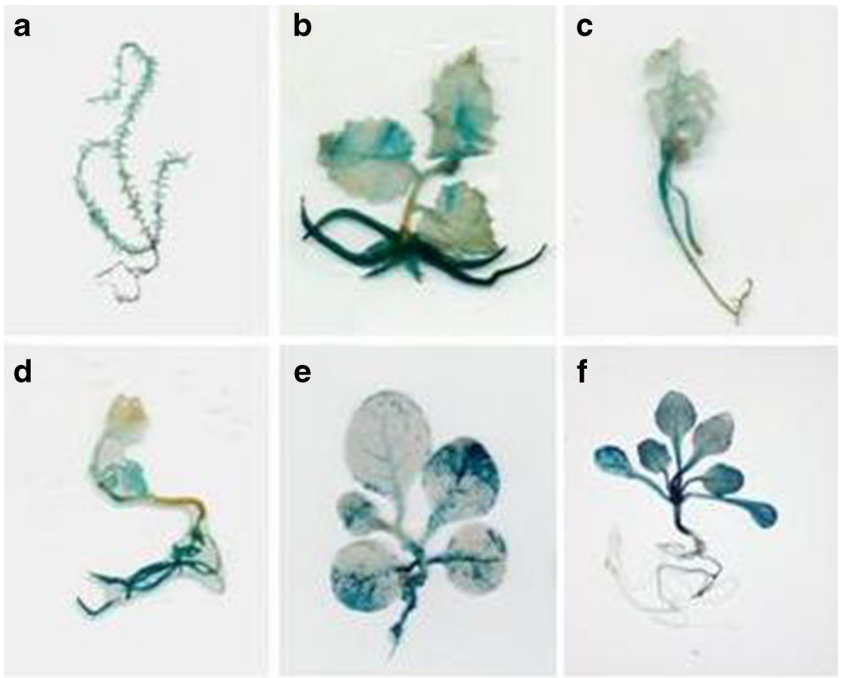

Fig. 5 GUS histochemical analyses of the effects of transient expression in different host plant species: **a** tamarisk (*Tamarix hispida*), **b** birch (*Betula platyphylla*), **c** willow (*Salix matsudana*), **d** aralia (*Aralia mandshurica*), **e** tobacco (*Nicotiana tabacum* Linn.), **f** *Arabidopsis thaliana* (L.) Heynh. GUS staining was performed after transformation for 72 h

genetic studies in plants without a stable transformation system, because it is suitable for various types of plants (Fig. 5). In addition, a quick way to determine the stress tolerance of a gene was also developed based on this transient transformation (Figs. 2, 3, 4), which allows the accurate analysis of the function of genes involved in stress response in a short time. Therefore, this transiently transformation method may be a powerful tool for characterizing gene function in plant species that lack stable transformation systems.

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Author Contributions Statement Y.C. Wang and X.Y. Ji conceived the project and designed experiments. X.Y. Ji, L. Zheng, Y.J. Liu, X.G. Nie and S.N. Liu participated in the experiments. X.Y. Ji performed the data analysis. X.Y. Ji, and Y.C. Wang drafted the manuscript. Y.C. Wang provided reagents and analysis tools.

Conflict of Interest Disclosure The authors declare that they have no conflict of interest

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