



# Overexpression of $\text{Na}^+/\text{H}^+$ antiporter gene *AtNHX1* from *Arabidopsis thaliana* improves the salt tolerance of kiwifruit (*Actinidia deliciosa*)

N. Tian<sup>a</sup>, J. Wang<sup>b</sup>, Z.Q. Xu<sup>a,\*</sup>

<sup>a</sup> Provincial Key Laboratory of Biotechnology of Shaanxi Province, Key Laboratory of Resource Biology and Biotechnology in Western China (Ministry of Education), Institute of Life Science, Northwest University, Xi'an, Shaanxi 710069, People's Republic of China

<sup>b</sup> College of Agriculture and Life Sciences, Ankang University, Shaanxi, Ankang 725000, People's Republic of China

Received 13 December 2009; received in revised form 21 July 2010; accepted 22 July 2010

## Abstract

Salinity is the main limiting factor of plant growth and agricultural productivity. A lot of previous works showed that the introduction of  $\text{Na}^+/\text{H}^+$  antiporter gene could improve the tolerance of plants to salt. In this study, a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter gene, *AtNHX1* from *Arabidopsis*, was transferred into kiwifruit by *Agrobacterium*-mediated protocol. Polymerase chain reaction (PCR) and Southern blot analysis confirmed that *AtNHX1* was successfully integrated into the kiwifruit genome. Reverse transcription (RT)-PCR analysis indicated that *AtNHX1* expressed highly in transgenic plants. It was found that transgenic kiwifruit plants exhibited improved resistance to 200 mmol/l NaCl in comparison with wide-type plants. Under salt stress, these transgenic lines accumulated more  $\text{Na}^+$  than control, due to an increased  $\text{Na}^+/\text{H}^+$  antiporter activity. In physiological analysis, the traits such as osmotic adjustment and antioxidation capability of transgenic lines under salt stress were obviously higher than that of wide-type plants. These results suggested that the overexpression of vacuolar  $\text{Na}^+/\text{H}^+$  antiporter gene could increase the salt tolerance of kiwifruit. © 2010 SAAB. Published by Elsevier B.V. All rights reserved.

**Keywords:** *Actinidia deliciosa*; *Agrobacterium tumefaciens*; *AtNHX1*; Salt tolerance

## 1. Introduction

The genus *Actinidia* Lindl. is large, containing 50 to 70 species of climbing plants originating mainly in southern China (Ferguson, 2007), since ancient times people of that area have known of the very delicious flavor of kiwifruit. Kiwifruit has been used in Chinese herbal remedy and Chinese folk medicine for treatment cancer since prerecorded times, including stomach, lung and liver cancer (Motohashi et al., 2002). Over the past 30 years, kiwifruit has developed into an important horticultural fruit tree, firstly in New Zealand, and subsequently in many different parts of the world such as Chile, China and Italy (Ferguson and Huang, 2007; Nishiyama, 2007). In recent years, it attracts more preference for as much as its distinctive flavors, pleasant fragrance, healthful components and potential medical prospects.

Kiwifruit is one of the most nutrient-dense fruits and is a good source of vitamin C, low in fat and rich in dietary fibre and phytochemicals (Nishiyama et al., 2004). It is currently accepted that kiwifruit has a preventive effect against certain cancers and cardiovascular diseases such as hypertension, hyperlipidemia, arteriosclerosis and diabetes (Du et al., 2009; Jang et al., 2008), which are thought to be in correlation with its phytosubstances and diverse constituents' ability in quenching active free radical oxygens, such as carotenoids and flavonoids (Kaur and Kapoor, 2001). The predominated flavonoid in kiwifruit is flavonol (Webby et al., 1994), and the main component in *Actinidia deliciosa* is rutin (Quercetin-3-rhamnoglucoside) (Greaves et al., 2001). In addition, the flower and fruit of kiwifruit contain many unique aroma compounds derived from different genetic pathways with potential for exploitation as nutraceuticals or medicine (Jordan et al., 2002).

Soil salinity is one of the major environmental factors limiting agricultural productivity in many regions of the world, because most crops are glycophytes and usually salt sensitive

\* Corresponding author.

E-mail address: [ziquinxu@nwu.edu.cn](mailto:ziquinxu@nwu.edu.cn) (Z.Q. Xu).

(Xue et al., 2004). Salinity imposes two types of stress on plant tissues, which sequentially affect plant on many aspects, including mineral and water uptake, enzyme activities, photosynthesis and metabolism (Takahashi et al., 2009), one is the water deficit resulting from the relatively high solute concentrations of the soil, the other is ion-specific stresses resulting from altered  $K^+/Na^+$  ratios,  $Na^+$  and  $Cl^-$  concentrations that are inimical to plants (Blumwald et al., 2000). Kiwifruit belong to glycophyte, and the soils they can grow are limited. The aim of our experiment is to alter the behavior and enhance the adaptability of kiwifruit to saline soil.

$Na^+$  is not essential for plant growth, although it is required in some plants, particularly halophytes. Halophytes have achieved salt-tolerant capability in the long evolution process to adapt themselves to saline environment. In salinity-stressed plants,  $Na^+/H^+$  antiporters play a key role in maintenance of the cytoplasmic  $K^+/Na^+$  ratio, through pumping  $Na^+$  either out of cells (sodium extrusion) or into organelles, mainly vacuole (sodium compartmentation), in exchange for  $H^+$ . From the first identification of the activity of  $Na^+/H^+$  antiporter in barley root tips in 1976 (Ratner and Jacoby, 1976), many  $Na^+/H^+$  antiporter genes have been characterized in plants, such as *Arabidopsis thaliana* (Apse et al., 1999), *Oryza sativa* (Fukuda et al., 1999; Gaxiola et al., 1999), *Atriplex gmelini* (Hamada et al., 2001), *Mesembryanthemum crystallinum* (Chauhan et al., 2000), *Suaeda salsa* (Ma et al., 2004), *Beta vulgaris* (Xia et al., 2002), *Gossypium hirsutum* (Wu et al., 2004) and *Trifolium repens* L. (Tang et al., 2009). The overexpression of  $Na^+/H^+$  antiporter genes could increase tolerance under saline conditions in transgenic *Arabidopsis* (Apse et al., 1999), tomato (Zhang and Blumwald, 2001), *Brassica* (Zhang et al., 2001), rice (Chen et al., 2007; Ohta et al., 2002), wheat (Xue et al., 2004), buckwheat (Chen et al., 2008) and *Petunia hybrida* (Xu et al., 2009). These works demonstrated that  $Na^+/H^+$  antiporter genes were crucial to plant salt-tolerance, and they could be used in crop improvement through genetic transformation.

Many crops have undesirable traits, such as salt sensitivity, thus improvement of their salt tolerance has been a tough task all the while in traditional breeding. The genetic improvement of tolerance by traditional cross-breeding has been hindered by the gynodioecious characteristics, genetic heterogeneity, and long life cycle of kiwifruit (Nakamura et al., 1999). Modern biotechnology creates a new approach for breeding with the development of tissue culture and transformation system. Several reports have been published in which gene function has been tested in transgenic kiwifruit (*Actinidia deliciosa*) transformed by *Agrobacterium*. The expression of  $\beta$ -1, 3-endoglucanase gene from soybean in kiwifruit increased resistance of young leaves to *Botrytis cinerea* (Nakamura et al., 1999). The transgenic kiwifruit harbouring rice homeobox-containing gene *OSH1* showed morphological changes including dissected leaf margins and dwarfism (Kusaba et al., 1999). Kobayashi et al. (2000) reported that expression of stilbene synthase gene from *Vitis* spp. led to production of resveratrol-glucoside in leaves of kiwifruit.

In the present work, *AtNHX1* driven by a constitutive promoter was transferred into kiwifruit on the basis of a high-

frequent regeneration system established by Tian et al. (2007). Several physiological parameters were examined to analyze the effect of salt stress on biomass production and the patterns of  $Na^+$  and  $K^+$  accumulation in transgenic plants. Our results confirmed that the biomass of *AtNHX1* transgenic kiwifruit was improved under saline condition compared to wild-type plants.

## 2. Materials and methods

### 2.1. Plant materials and expression vector

The stems of kiwifruit genotype ‘Qin mei’ were collected from Hu county of Shaanxi province. Plant expression vector pHZX1 containing a selectable marker gene *NPTII* and  $Na^+/H^+$  antiporter gene *AtNHX1* was kindly provided by Dr. HX Zhang of the National Institute of Plant Physiology in Shanghai. *AtNHX1* is controlled by cauliflower mosaic virus (CaMV) 35S promoter and 3'-UTR, polyadenylation signal and terminator region of nopaline synthetase gene (Nos).

### 2.2. Callus induction and plant regeneration

The stems were washed under running water for 6 h, followed by surface-sterilization with 75% ethanol for 3 min and 0.1%  $HgCl_2$  for 8 min. After rinsing for 3 times with sterile distilled water, the stems were cut into 0.5 cm segments and inoculated on MS medium supplemented with 2.0 mg/l 6-BA, 1.0 mg/l NAA, 600 mg/l casein hydrolysate, 500 mg/l yeast extract for callus induction and regeneration under 2000 lx illumination, 16 h photoperiod and  $25 \pm 2^\circ C$ . Calli and regenerated shoots were subcultured at regular intervals of 3 weeks. The regenerated shoots of 2.0 cm high were rooted on half MS medium (Murashige and Skoog, 1962) containing 1.0 mg/l IBA. All media contained 30 g/l sucrose and were solidified with 0.7% agar. The pH was adjusted to 5.8–6.2 prior to autoclaving at  $121^\circ C$  for 25 min.

### 2.3. Transformation of the explants

*Agrobacterium* strain LBA4404 was inoculated into YEB liquid medium (5.0 g/l tryptone, 5.0 g/l sucrose, 1.0 g/l yeast extract, 0.5 g/l  $Mg_2SO_4 \cdot 7H_2O$ , 50 mg/l kanamycin and 100 mg/l streptomycin) and incubated at  $28^\circ C$  with constant shaking (200 rpm) overnight. One millilitre bacterium suspension was transferred into 100 ml YEB liquid medium and cultured for 5 h until the density of *Agrobacterium* ( $OD_{600}$ ) achieved 0.5. Then the bacteria were harvested by centrifugation at 3000 rpm for 10 min and resuspended in MS liquid medium, the  $OD_{600}$  was adjusted to 0.2–0.8. Stem and leaf explants precultured for 0–4 days were immersed in *Agrobacterium* suspension for 5–25 min in the dark. Excess bacteria on the explant surface were removed with sterilized filter paper and the explants were co-cultured at  $25^\circ C$  in the dark for 1–4 days. Acetosyringone (AS) was added at a different concentration (0–250  $\mu mol/l$ ) to the medium during the period of coculture. The infected explants were rinsed with sterile water more than 3 times, and subcultured on selection and regeneration medium supplemented with

20 mg/l kanamycin, 500 mg/l cefotaxime at 3 weeks intervals. Approximately 8 weeks later, survived shoots were transferred onto half MS medium containing 1.0 mg/l IBA to induce roots. Transformation efficiency is usually expressed as the number of explant with kanamycin-resistant calli/initial number of explant inoculated.

#### 2.4. PCR and Southern blotting analysis

Genomic DNA was extracted from leaf tissue of kanamycin-resistant plants using the cetyl trimethyl ammonium bromide (CTAB) method. The presence of *AtNHX1* in putative transgenic plants was analyzed by PCR with a primer pair of 5'-CACTCACCTAAACCACGAAGC-3' and 5'-CAGACCACCAAATCACAACC-3'. The amplification conditions used were 10 min predenaturation at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min, and a 10 min final extension step at 72 °C.

PCR positive plants were further confirmed by Southern blotting analysis. The 560-bp fragment amplified from *AtNHX1* was purified and used as probe. Twenty microgram of genomic DNA digested with *EcoRI* or with *HindIII* and *EcoRI*, respectively, were separated by electrophoresis in 1.0% agarose gel. After transferred to nylon membrane, genomic DNA was hybridized with *AtNHX1* probe using DIG random labeling and detection system (Roche).

#### 2.5. Expression of *AtNHX1* in transgenic kiwifruit

Reverse transcription PCR (RT-PCR) was carried out to measure the expression level of *AtNHX1* in transgenic lines. Total RNA was isolated according to the mini-prep procedure described previously (Wilkosz and Schläppi, 2000) and treated with RNase-free DNase I (Takara). One microgram of total RNA was added in 10 µl RT reaction systems with oligo dT as primer and cDNAs were synthesized with Reverse Aid First Strand cDNA Synthesis Kit (Fermantas). One microlitre of the RT reaction mixture was used as template in amplification of *AtNHX1* in a 20 µl PCR system and the conditions were same as above-mentioned.

#### 2.6. Determination of $\text{Na}^+$ , $\text{K}^+$ , proline and malondialdehyde contents in transgenic kiwifruit

Two transgenic lines (TL1 and TL2) and wild-type kiwifruit were cultured on MS medium for 2 weeks and then transferred to MS medium containing different concentrations of NaCl (0, 100 and 200 mmol/l respectively) for 3 days. Roots were rinsed with distilled water to remove NaCl on the surface, and then the leaves and roots of each treatment were harvested and dried at 60 °C overnight.  $\text{Na}^+$  and  $\text{K}^+$  concentrations were measured by a flame photometer (Thermo). Free proline content in leaves was determined according to the method given by Bates et al. (1973). The levels of lipid peroxidation in fresh leaves, stems and roots were measured in terms of malondialdehyde (MDA)

content by thiobarbituric acid (TBA) reaction method (Karadeniz et al., 2005).

#### 2.7. Determination of flavonoids in transgenic kiwifruit

5% NaOH was employed to stain the flavonoids distributed in different tissues of plant (Tan et al., 2007). A modified spectrophotometric method was used to determine the flavonoid contents of transgenic kiwifruit. Dry samples were grinded to fine powder in a mortar and pestle, then immersed in methanol (0.1 g/20 ml), and treated with ultrasonic wave for 30 min. The samples were centrifuged at 12,000 rpm for 20 min. A 0.5 ml aliquot of the samples was transferred to a test tube and 3 ml of 30% ethanol and 0.3 ml of 5% sodium nitrite were added. The solution was mixed thoroughly and stood at room temperature for 5 min followed by addition of 0.3 ml of 10% aluminium chloride. After 6 min, 2 ml of 1 M sodium hydroxide was added to the test tube. The solution was then diluted with 30% ethanol to make the final volume up to 10 ml. The absorbance at 510 nm was checked and the flavonoid content was calculated with a standard calibration curve prepared with rutin (Subhasree et al., 2009).

### 3. Results

#### 3.1. Effects of preculture, infection and coculture time on transformation frequency of kiwifruit

As shown in Fig. 1A, the transformation frequencies changed with explant type and preculture time. The result indicated that the stem was much more suitable for transformation by *Agrobacterium tumefaciens* than the leaf, presumably the leaf was vulnerable. Transformation frequency of leaf explants significantly increased if they were precultured for 1–2 days. On the contrary, preculture had no effect in stem transformation. However, the transformation frequencies decreased after 4 days preculture.

Time of *Agrobacterium* infection and coculture were crucial to transformation. The result (Fig. 1B and C) demonstrated that the appropriate infection time and coculture time for different explants were variable. The proper infection time was 2 min for leaf and 15 min for stem. Two days coculture with *Agrobacterium* was better for both stem and leaf. If the coculture time exceeded three days, the explants would be injured seriously by *Agrobacterium*. It is possible that leaves are more fragile to *Agrobacterium* than stems, which led to the differences between stem and leaf on infection and coculture time.

#### 3.2. Effects of *Agrobacterium* density and AS on transformation of kiwifruit

The density of *Agrobacterium* influenced the transformation frequency obviously. Suitable densities of *Agrobacterium* not only facilitated the infection process of *Agrobacterium*, but also reduced its detrimental effect to explants. As shown in Fig. 1D, the fragile leaves required a low density of *Agrobacterium* (optimal  $\text{OD}_{600}=0.2$ ) compared to stems (optimal  $\text{OD}_{600}=0.6$ ).

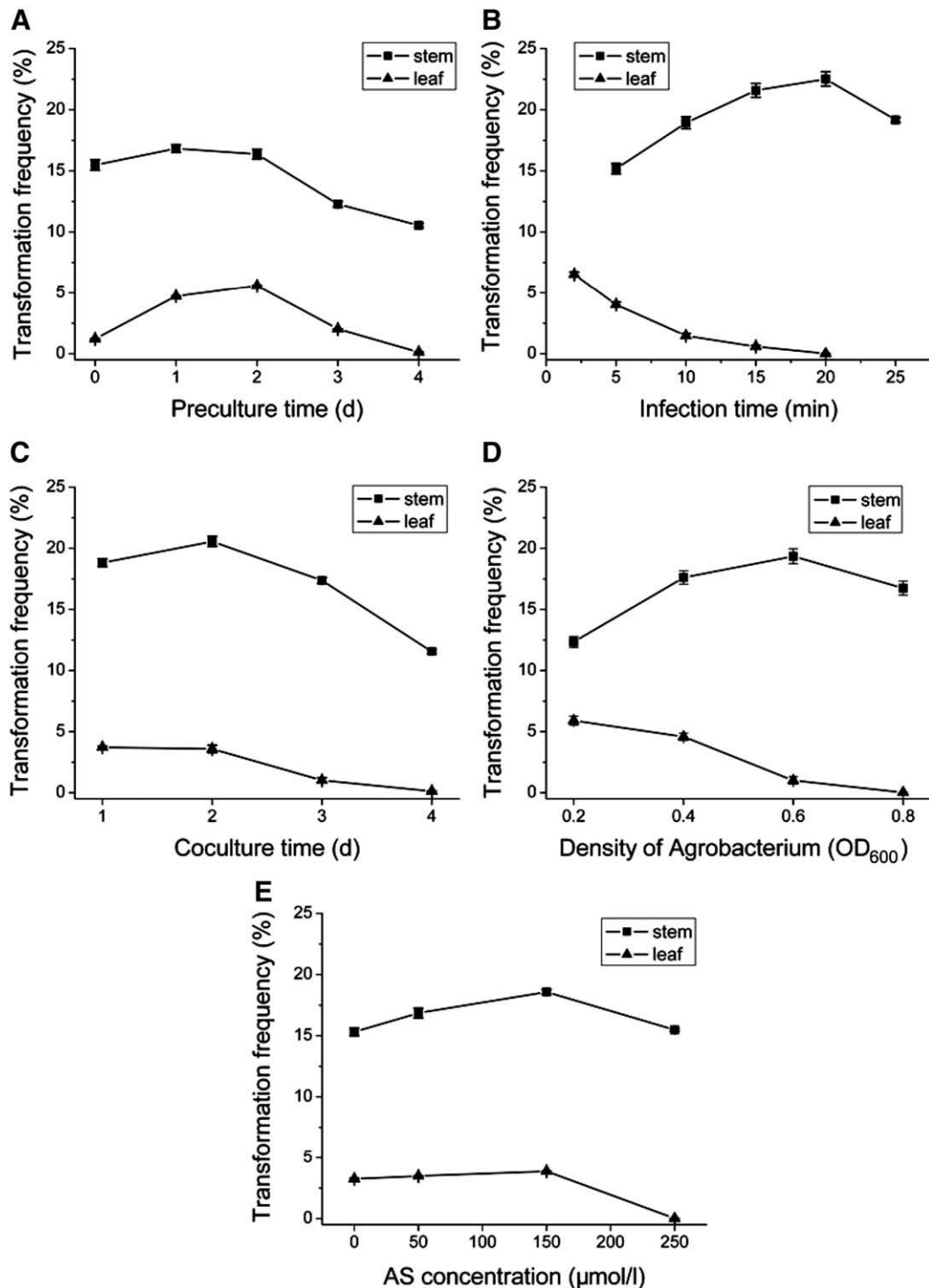


Fig. 1. The effect of preculture time (A), infection time (B), coculture time (C), *Agrobacterium* density (D) and acetosyringone (E) on transformation of kiwifruit.

Acetosyringone (AS) is regarded as one of the most effective signal molecules, with the function in activating expression of *vir* genes (Berthelot et al., 1998). It could significantly increase the transformation frequency in certain plant species (Ozawa, 2009), especially in monocotyledons which are insensitive to *Agrobacterium*. However, high concentration of AS is toxic to explants. To promote the transformation frequency, AS was added to the medium during the period of coculture. The effect of

AS on transformation frequency was slight (Fig. 1E), so it was unnecessary to add AS in the process of kiwifruit transformation.

### 3.3. Regeneration of transgenic kiwifruit

The explants after 1–2 days coculture with *Agrobacterium* were selected on the regeneration medium containing kanamycin. 4 weeks later, most explants browned, only 17% of them



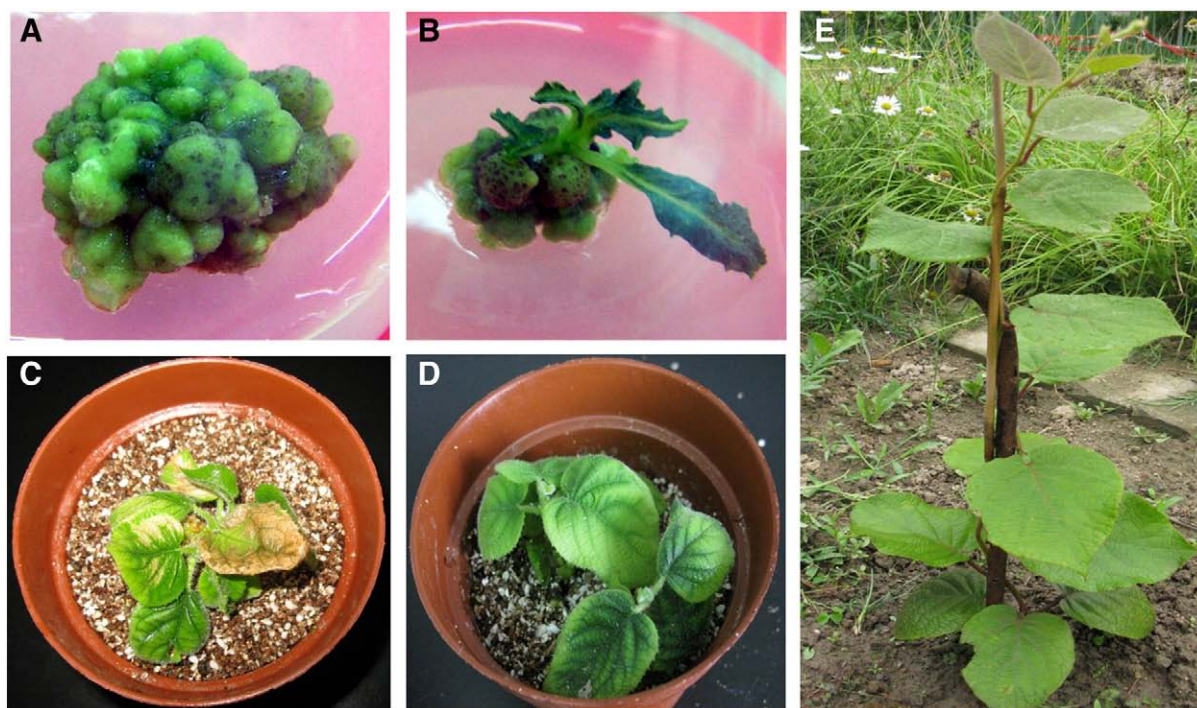


Fig. 2. Plant regeneration of transgenic kiwifruit. Calli induced on MS medium containing 2 mg/l 6-BA and 1 mg/l NAA (A). Adventitious shoots regenerated from kanamycin-resistant calli (B). Transgenic (D) and wild-type (C) plants stressed by 200 mmol/l NaCl for two weeks. One year transgenic plant transferred to soil (E).

produced green calli (Fig. 2A). Subcultured green calli produced shoots in 4 weeks (Fig. 2B). The regenerated shoots produced roots on rooting medium in one month. Strong plantlets were exercised and transplanted to soil (Fig. 2E).

### 3.4. Molecular identification of transgenic plants

4 transgenic lines of kiwifruit were identified by PCR amplification of a 560 bp fragment of *AtNHX1* gene (TL1–TL4,

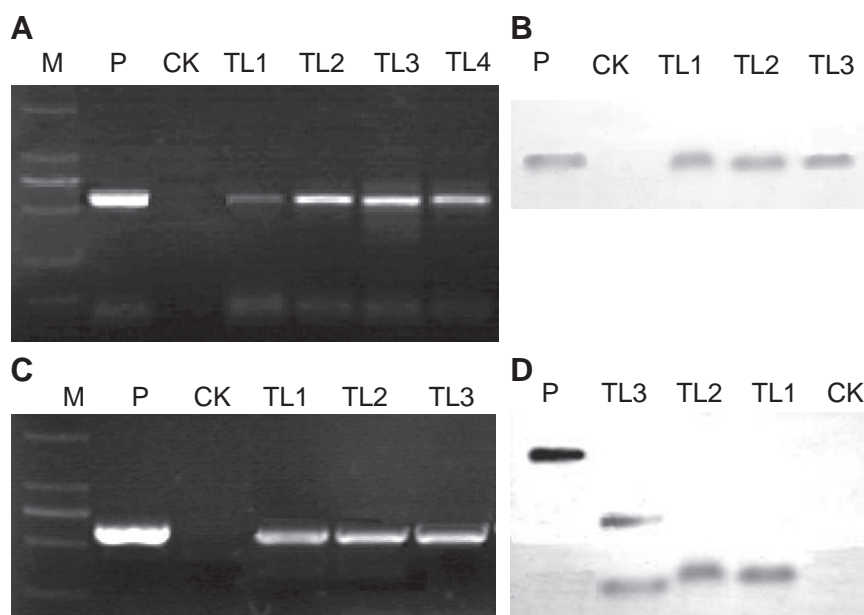


Fig. 3. Molecular identification of transgenic kiwifruit lines. (A) PCR analysis of the presence of *AtNHX1*. P, pHZX, positive control; CK, wild-type kiwifruit, negative control; lanes 1, 2, 3 and 4, four putative transgenic lines. (B) Southern blotting analysis of the transgenic kiwifruit lines. P, plasmid pHZX1 digested with *EcoRI*/*HindIII*; CK, genomic DNA of wild-type plant digested with *EcoRI*/*HindIII*; L1, L2, L3, genomic DNA of three independent transgenic lines digested by *EcoRI*/*HindIII*. (C) RT-PCR analysis of the transgenic kiwifruit lines. P, pHZX; CK, wild-type of kiwifruit; L1, L2, two independent transgenic lines. (D) Southern blotting analysis of the transgenic kiwifruit lines. P, plasmid pHZX1 digested with *EcoRI*; CK, genomic DNA of wild-type plant digested with *EcoRI*; L1, L2, L3, genomic DNA of three independent transgenic lines digested by *EcoRI*.

Fig. 3A). Three independent transgenic lines (TL1–TL3) were selected for Southern blotting analysis. When the DNA were double digested with *Hind*III and *Eco*RI, the expected 1.9 kbp band (corresponding to the fragment containing CaMV 35 S promoter, the Na<sup>+</sup>/H<sup>+</sup> antiporter cDNA and Nos terminator) was detected in all of the PCR positive plants (Fig. 3B, lanes TL1 to TL3), but not in the control plant (Fig. 3B, lane CK). To confirm the integration sites of the cDNA fragment in the transgenic plant genome, the genome DNA were single-digested with *Eco*RI because pHZX has a single restriction site of *Eco*RI. The transgenic plants showed one to two bands which represented junctions between T-DNA and adjacent plant DNA (Fig. 3D). These findings showed TL1 and TL2 possessed a single copy of *AtNHX1*, while TL3 has two copies. TL1 and TL2 lines were further analyzed on the transcription level by RT-PCR. As show in Fig. 3C, *AtNHX1* was expressed effectively in TL1 and TL2.

### 3.5. Determination of Na<sup>+</sup>, K<sup>+</sup>, proline and malondialdehyde contents of transgenic kiwifruits

Vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters catalyze the exchange of Na<sup>+</sup> for H<sup>+</sup> across vacuolar membranes, and compartmentalize Na<sup>+</sup> into

vacuoles (Glenn et al., 1999). Sodium and potassium play important roles in salt stressed plants. To determine if over-expression of *AtNHX1* could increase Na<sup>+</sup> accumulation in kiwifruit, Na<sup>+</sup> and K<sup>+</sup> contents were examined in transgenic and wild-type plants. Physiological analysis was carried out in two transgenic lines TL1 and TL2 to evaluate the salt tolerance. Without salt stress, those contents were nearly the same in both transgenic and wild-type plants. Na<sup>+</sup> contents in leaves and roots of the transgenic lines and wild-type kiwifruit both increased with the rising NaCl concentration (100–200 mmol/l). However, at the range of 100–200 mmol/l NaCl, Na<sup>+</sup> contents in leaves (Fig. 4A) and roots (Fig. 4C) of transgenic lines were higher than that of the wild-type plants, especially at 200 mmol/l NaCl concentration. The result demonstrated that the transgenic lines accommodated more quickly to Na<sup>+</sup> stress than wild-type plant. The reason might be efficient expression of Na<sup>+</sup>/H<sup>+</sup> antiporter gene *AtNHX1* in transgenic lines could sequester Na<sup>+</sup> and compartmentalize it to vacuole to keep a high cytosolic K<sup>+</sup>/Na<sup>+</sup> ratio.

The K<sup>+</sup> contents in leaves and roots of transgenic lines and wild-type kiwifruit both decreased with the increase of NaCl concentration (Fig. 4B and D). Whereas, K<sup>+</sup> contents in leaves and roots of transgenic lines were significantly higher than that

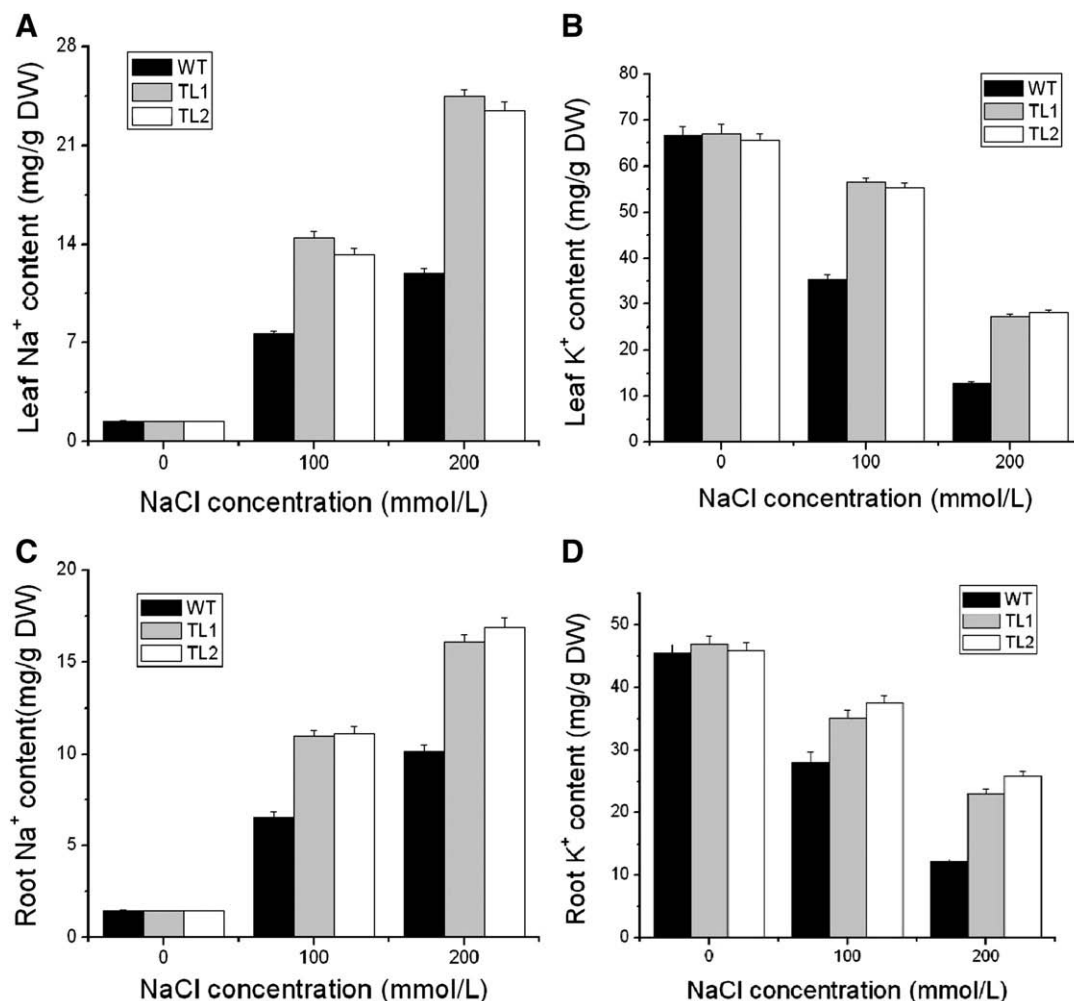


Fig. 4. Na<sup>+</sup>, K<sup>+</sup> contents in leaves (A, B) and roots (C, D) of transgenic lines and the wild-type kiwifruit. Error bars (n=3) ± S.D. are shown.

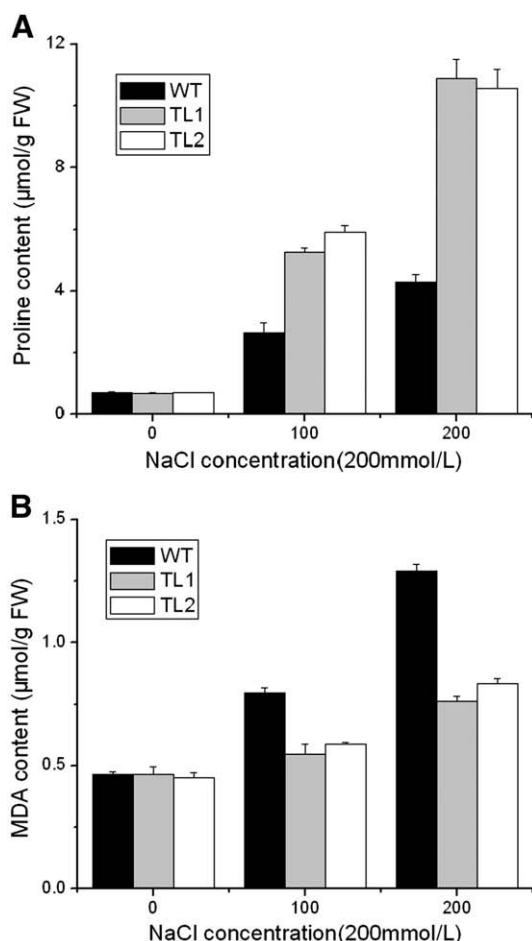


Fig. 5. Proline (A) and MDA (B) contents in leaves of transgenic lines and wild-type kiwifruit stressed by NaCl. Error bars ( $n=3$ )  $\pm$  S.D. are shown.

of wild-type plants at a high concentration of NaCl (200 mmol/l).  $K^+$  is the most abundant cation in plants, involved in many metabolism processes, such as root and shoot growth, tropisms, cell expansion, enzyme activity, ion homeostasis, stomatal movements and osmotic regulation (Glenn et al., 1999). Hence the transgenic kiwifruit lines could maintain high  $K^+/Na^+$  ratio in cytosol, so as to promote metabolism processes.

Proline can protect macromolecules from dehydration and be used as hydroxyl radical scavenger. When plants suffer from adversity, they usually produce abundant free proline to adjust osmotic-stress (Bates et al., 1973). After treated by NaCl, contents of free proline in leaves of transgenic lines were significantly higher than that of the wild-type plants (Fig. 5A). This result further confirmed the function of the  $Na^+/H^+$  antiporter in opposing to salt stress.

Lipid peroxidation is related to the oxidative degradation of polyunsaturated fatty acids and involves free radicals. This membrane damage process results in deleterious effects and produces malondialdehyde (MDA) (Glenn et al., 1999). MDA level under stress conditions could mirror the antioxidative capacity of plant cell. In the present work, the contents of MDA in the leaves of transgenic lines and wild-type plants were measured to depict the membrane lipid peroxidation status of them stressed by different NaCl concentrations. After treatment with NaCl, the MDA contents increased in the transgenic lines and wild-type kiwifruit. However, in each concentration the MDA contents of transgenic lines were less than that of the wild-type plants after 3 d treatment with NaCl (Fig. 5B). These data indicated that overexpression of  $Na^+/H^+$  antiporter gene *AtNHX1* was correlated with the alleviation of the detrimental effect of  $Na^+$ .

Transgenic lines (TL1 and TL2) and wild-type plants were transferred to soil and grown for two weeks, then watered with nutrient solution containing 0–200 mmol/l NaCl. After 20 days, obvious salt stress effect on the growth of the transgenic plants and control could be observed. The leaves of the wild-type plants gradually turned yellow and withered after two weeks when the concentration of NaCl reach 200 mmol/l, whereas transgenic lines still kept green and grown (Fig. 2C, D). The inhibition of NaCl on vegetative growth was shown in Table 1. It demonstrated that the growth status of the transgenic lines obviously excelled the control plants. The transgenic lines were less-inhibited by salt stress compared to wild-type plants further confirmed that the overexpression of the  $Na^+/H^+$  antiporter gene could indeed improve salt tolerance.

### 3.6. Determination of flavonoids in different tissues of transgenic kiwifruit

Flavonoids take part in prohibiting cell aging and antagonizing ROS, especially when plants are stressed by salt, drought, pathogen, ultraviolet and other adverse factors (Glenn et al., 1999). They are important secondary metabolites and nutrition components of kiwifruit. In the present work, antioxidant capacity of *AtNHX1* transgenic kiwifruit was analyzed in terms of flavonoids contents. To estimate the ability of reducing ROS of the transgenic kiwifruit, the flavonoids contents in leaves, stems and roots of transgenic lines were measured after treatment with 200 mmol/l NaCl for three days. As shown in Fig. 6, in different tissues of kiwifruit, the flavonoids contents of leaf were the highest and stem contained more flavonoids than root in general. The contents of flavonoids in transgenic lines were significantly higher than that of the control, especially in

Table 1  
Effects of NaCl on the vegetative growth of transgenic lines and wild-type kiwifruit.

NaCl concentration (mmol/L)	WT		TL1		TL2	
	0	150	0	150	0	150
Height (cm)	2.60 $\pm$ 0.14	1.03 $\pm$ 0.04	2.55 $\pm$ 0.07	2.05 $\pm$ 0.07	2.67 $\pm$ 0.10	1.83 $\pm$ 0.07
Fresh weight (g)	2.06 $\pm$ 0.02	0.64 $\pm$ 0.03	2.12 $\pm$ 0.05	1.64 $\pm$ 0.02	1.97 $\pm$ 0.04	1.44 $\pm$ 0.02



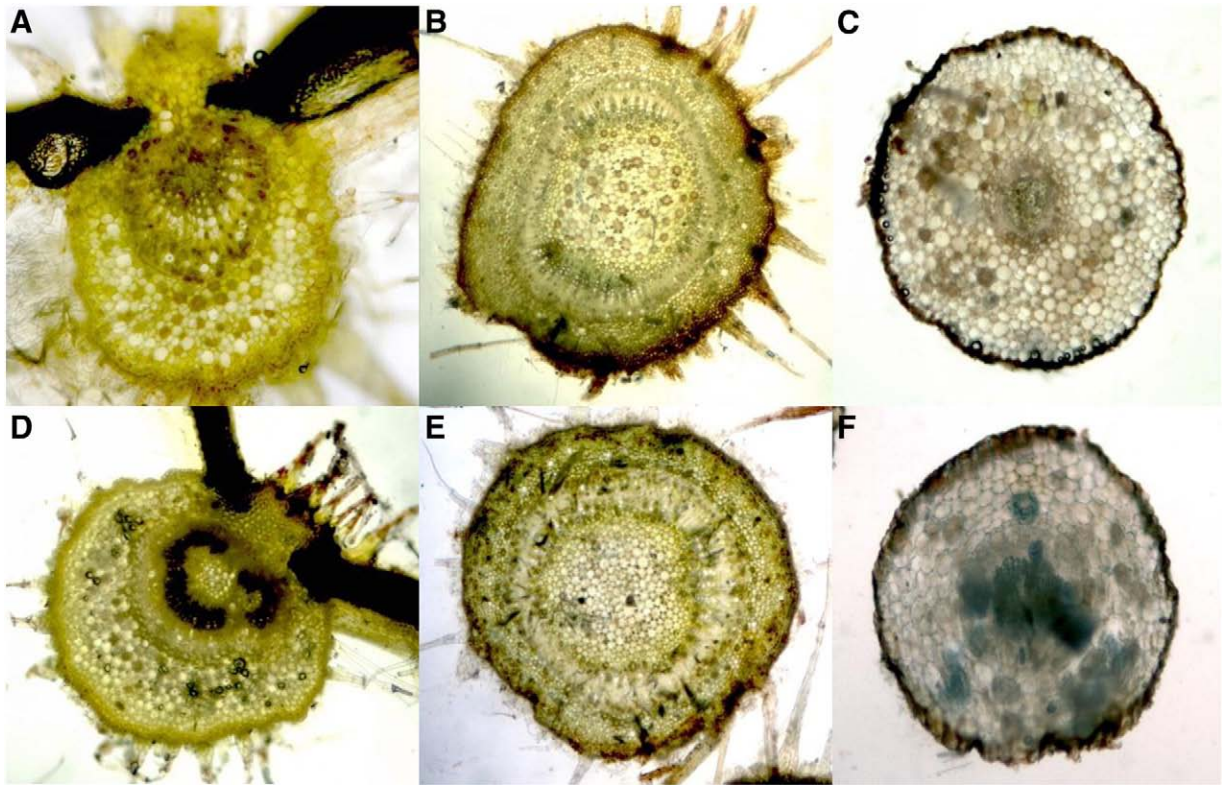


Fig. 6. Flavonoids distribution in leaf (A), stem (B) and root (C) of transgenic line (TL1) and in leaf (D), stem (E) and root (F) of wild-type kiwifruit.

leaves of transgenic lines. The flavonoid contents in whole plants of transgenic lines TL1 and TL2 increased 70.6% and 40.0% respectively compared to the wild-type plants (Table 2). The contents of flavonoids in leaves of transgenic lines TL1 and TL2 increased 68% and 44% respectively compared to that of wild-type plants (Table 2). All these results coincided with the change of MDA content and confirmed that the transgenic lines had higher ROS elimination activity.

#### 4. Discussion

*Agrobacterium*-mediated protocol is an effectual approach for transformation of plants, especially dicot, many scientifically and agronomically important species are routinely transformed using *Agrobacterium*. In this paper, we studied the factors affecting the transformation of *Actinidia deliciosa* mediated by *Agrobacterium*. We found that *Actinidia deliciosa* showed high sensitivity to *Agrobacterium* without the facilitation of AS, and the young stem was much more suitable explant

in comparison with young leaf, due to its better endurance to *Agrobacterium*, although they had a difference in regeneration capability. The damage of *Agrobacterium* to leaf was alleviated by lowering the density of *Agrobacterium* ( $OD_{600}=0.2$ ) and shortening the infection time (2 min). This phenomenon was also observed in *Agrobacterium*-mediated transformation of rice (Ozawa, 2009). Coculture of explants with *Agrobacterium* in the presence of AS, a *vir* gene inducer, has become a routine exercise in the transformation of recalcitrant crops such as rice, maize, barley and wheat (Bartels and Sunkar, 2005). However, influence of AS is trivial on promotion of *Actinidia deliciosa* transformation. The results showed that *AtNHX1*-positive transgenic plants could be regenerated effectively from stem co-cultured for 2 days with *Agrobacterium* ( $OD_{600}=0.2$ ) and after infection for 15 min.

High-salt stress is one of the major adverse environmental conditions that affect plant growth, development and crop yield. To avoid salt damage, plants have evolved different mechanisms to limit  $Na^+$  uptake or compartmentalize  $Na^+$  into vacuoles, and  $Na^+/H^+$  antiporters play a key role in the maintenance of osmotic balance (Bartels and Sunkar, 2005). Recently, great progress of improving plant salt tolerance has been made through adopting the strategies of reestablishing ion homeostasis, such as manipulating plant vacuolar  $Na^+/H^+$  antiporter to produce transgenic plants. Overexpression of plant vacuolar  $Na^+/H^+$  antiporter gene, *AtNHX1* from *Arabidopsis*, in several dicotyledonous and monocotyledonous species, including tomato, Brassica, rice, wheat, and other crops, enable transgenic plants to grow in high concentration of salt,

Table 2  
Comparison of flavonoids contents in transgenic lines and wild-type kiwifruit treated with NaCl.

Materials	Flavonoid content ( $\mu\text{g/g DW}$ )		
	Leaf	Stem	Root
WT	$15.45 \pm 0.52$	$5.26 \pm 0.46$	$1.20 \pm 0.20$
TL1	$25.94 \pm 0.94$	$7.18 \pm 0.71$	$1.99 \pm 0.28$
TL2	$22.27 \pm 0.72$	$6.89 \pm 0.65$	$1.73 \pm 0.22$



demonstrating the feasibility of producing salt-tolerant crop plants via introducing plant vacuolar  $\text{Na}^+/\text{H}^+$  antiporter into aimed plants (Yamaguchi and Blumwald, 2005). We obtained similar results in kiwifruit, further verifying the importance of *AtNHX1* in salt tolerance.

Intracellular high concentration  $\text{K}^+$  and low concentration  $\text{Na}^+$  are important for the activities of many cytosolic enzymes and for maintaining membrane potential and an appropriate osmoticum for cell volume regulation. Under salt stress, the rate of transporter can be affected by excessive  $\text{Na}^+$  in cytosol through its competition for  $\text{K}^+$  binding sites of  $\text{K}^+$  transporters such as AKT1 (Zhu, 2003). In this study, it was also found that  $\text{Na}^+$  and  $\text{K}^+$  accumulation in leaves of the transgenic kiwifruit were higher than that of wild-type plants under severe saline conditions (200 mmol/l NaCl), and maintained a higher  $\text{K}^+/\text{Na}^+$  ratio. The transgenic plant cells obtained the enhanced ability to efficiently sequester excessive  $\text{Na}^+$  into vacuole and decrease the  $\text{Na}^+$  concentration in cytosol. It thus not only averts ion toxic effect on cytosolic enzymes and plasma membrane but also maintains higher  $\text{K}^+$  concentration through alleviating the inhibition of  $\text{K}^+$  uptake. These results indicated that *AtNHX1* overexpression increased the plant salt tolerance by elevating  $\text{Na}^+$  compartmentalization and keeping the  $\text{K}^+/\text{Na}^+$  balance mainly in the leaves.

A remarkable improvement in biomass production at the vegetative growth stage was observed between the transgenic lines and wild-type kiwifruit under salt stress. In the present work, the fresh shoot weight of wild-type plants was 31% of the non-saline condition with the treatment of 150 mmol/l NaCl for 20 days, in comparison with that of the transgenic line TL1, 77% of the non-saline control, which was two times higher than the wild-type plants. The growth reduction of kiwifruit in saline conditions is mainly contributed to the  $\text{Na}^+$ -specific toxicity and nutrient imbalance. Improved growth of the transgenic plants might be the result of the transporter compartmentalizing  $\text{Na}^+$  into the vacuole and maintaining high level  $\text{K}^+$  in cytosol.

The concept of using physiological criteria to improve the growth and productivity in saline environment has been successfully assessed in breeding programs (Mansour et al., 2003). In the present work, several physiologic parameters of transgenic lines were analyzed. Under salt stress, the transgenic lines showed better osmotic adjustment, higher antioxidant capacity and lower membrane damage and more vigorous growth than wild-type plants. Apparently, no toxicity and extra detrimental metabolic burden were observed for the overexpression of *AtNHX1* in kiwifruit using the constitutive CaMV 35S promoter. These findings demonstrate that *AtNHX1* can be useful for practical applications with cultivated crops. However, further field tests at various sites are necessary for confirmation of the commercial viability of these *AtNHX1* kiwifruit lines.

## Acknowledgements

We thank Hongxia Zhang for providing pHZX1 plasmid and Yafu Zhou for help with showing flavonoids in tissue sections. This work was supported by the National Natural Science Foundation of China (Grant number: 30870194), the Research

Project of Provincial Key Laboratory of Shaanxi (Grant number: 08JZ70), Development Project of Science and Technology Research of Shaanxi Province (the Program for Tackling Key Problems) (Grant number: 2010 K16-04-01) and the Postgraduate Innovation Research Project of Northwest University (09YZZ58, 09YSY38).

## References

- Apse, M.P., Aharon, G.S., Snedden, W.A., Blumwald, E., 1999. Salt tolerance conferred by overexpression of a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter in *Arabidopsis*. *Science* 285, 1256–1258.
- Bartels, D., Sunkar, R., 2005. Drought and salt tolerance in plants. *Critical Reviews in Plant Sciences* 24, 23–58.
- Bates, L.S., Waldren, R.P., Teare, J.D., 1973. Rapid determination of proline for water stress studies. *Plant and Soil* 39, 205–207.
- Berthelot, K., Buret, D., Guérin, B., Delay, D., Negrel, J., Delmotte, F.M., 1998. *Vir*-Gene-inducing activities of hydroxycinnamic acid amides in *Agrobacterium tumefaciens*. *Phytochemistry* 49, 1537–1548.
- Blumwald, E., Aharon, G.S., Apse, M.P., 2000. Sodium transport in plant cells. *Biochimica et Biophysica Acta: Biomembranes* 1465, 140–151.
- Chauhan, S., Forsthoefel, N., Ran, Y., Quigley, F., Nelson, D.E., Bohnert, H.J., 2000.  $\text{Na}^+/\text{myo-inositol}$  symporters and  $\text{Na}^+/\text{H}^+$ -antiporter in *Mesembryanthemum crystallinum*. *The Plant Journal* 24, 511–522.
- Chen, H., An, R., Tang, J.H., Cui, X.H., Hao, F.S., Chen, J., Wang, X.C., 2007. Over-expression of a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter gene improves salt tolerance in an upland rice. *Molecular Breeding* 19, 215–225.
- Chen, L.H., Zhang, B., Xu, Z.Q., 2008. Salt tolerance conferred by overexpression of *Arabidopsis* vacuolar  $\text{Na}^+/\text{H}^+$  antiporter gene *AtNHX1* in common buckwheat (*Fagopyrum esculentum*). *Transgenic Research* 17, 121–132.
- Du, G., Li, M., Ma, F., Liang, D., 2009. Antioxidant capacity and the relationship with polyphenol and Vitamin C in *Actinidia* fruits. *Food Chemistry* 113, 557–562.
- Ferguson, A.R., 2007. The need for characterisation and evaluation of germplasm: kiwifruit as an example. *Euphytica* 154, 371–382.
- Ferguson, A.R., Huang, H.W., 2007. Genetic resources of kiwifruit: domestication and breeding. In: Janick, J. (Ed.), *Horticultural Reviews*. John Wiley & Sons Inc., Hoboken, pp. 1–121.
- Fukuda, A., Nakamura, A., Tanaka, Y., 1999. Molecular cloning and expression of the  $\text{Na}^+/\text{H}^+$  exchanger gene in *Oryza sativa*. *Biochimica et Biophysica Acta* 1446, 149–155.
- Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L., Fink, G.R., 1999. The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proceedings of the National Academy of Sciences of the United States of America* 96, 1480–1485.
- Glenn, E.P., Brown, J.J., Blumwald, E., 1999. Salt-tolerant mechanisms and crop potential of halophytes. *Critical Reviews in Plant Sciences* 18, 227–255.
- Greaves, T.J., McGhie, T.K., Piller, G.J., Meekings, J.S., Reglinski, T., Klages, K., Boldingh, H.L., 2001. Carbon status during leaf development in kiwifruit and subsequent resistance of wounded tissue to *Botrytis cinerea*. *Crop Protection* 20, 553–560.
- Hamada, A., Shono, M., Xia, T., Ohta, M., Hayashi, Y., Tanaka, A., Hayakawa, T., 2001. Isolation and characterization of a  $\text{Na}^+/\text{H}^+$  antiporter gene from the halophyte *Atriplex gmelini*. *Plant Molecular Biology* 46, 35–42.
- Jang, D.S., Lee, G.Y., Kim, J., Lee, Y.M., Kim, J.M., Kim, Y.S., Kim, J.S., 2008. A new pancreatic lipase inhibitor isolated from the roots of *Actinidia arguta*. *Archives of Pharmacol Research* 31, 666–670.
- Jordan, M.L., Margaria, C.A., Shaw, P.E., Goodner, K.L., 2002. Aroma active components in aqueous kiwifruit essence and kiwifruit puree by GC–MS and multidimensional GC/GC–O. *Journal of Agricultural and Food Chemistry* 50, 5386–5390.
- Karadeniz, F., Burdurlu, H.S., Koca, N., Soyer, Y., 2005. Antioxidant activity of selected fruits and vegetables grown in Turkey. *Turkish Journal of Agriculture and Forestry* 29, 297–303.

- Kaur, C., Kapoor, H.C., 2001. Antioxidant in fruits and vegetables—the millennium's health. *International Journal of Food Science & Technology* 36, 703–725.
- Kobayashi, S., Ding, C.K., Nakamura, Y., Nakajima, I., Matsumoto, R., 2000. Kiwifruit (*Actinidia deliciosa*) transformed with a *Vitis* stilbene synthase gene produce piceid (resveratrol-glucoside). *Plant Cell Reports* 19, 904–910.
- Kusaba, S., Kano-Murakami, Y., Matsuoka, M., Matsuta, N., Sakamoto, T., Fukumoto, M., 1999. Expression of the rice homeobox gene, *OSH1*, causes morphological changes in transgenic kiwifruit. *Journal of the Japanese Society for Horticultural Science* 68, 482–486.
- Ma, X.L., Zhang, Q., Shi, H.Z., Zhu, J.K., Zhao, Y.X., Ma, C.L., Zhang, H., 2004. Molecular cloning, and different expression of a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter gene in *Suaeda salsa* under salt stress. *Biologia Plantarum* 48, 219–225.
- Mansour, M.M.F., Salama, K.H.A., Al-Mutawa, M.M., 2003. Transport proteins and salt tolerance in plants. *Plant Science* 164, 891–900.
- Motohashi, N., Shirataki, Y., Kawase, M., Tani, S., Sakagami, H., Satoh, K., Kurihara, T., Nakashima, H., Mucsi, I., Varga, A., Molnar, J., 2002. Cancer prevention and therapy with kiwifruit in Chinese folklore medicine: a study of kiwifruit extracts. *Journal of Ethnopharmacology* 81, 357–364.
- Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* 15, 473–497.
- Nakamura, Y., Sawada, H., Kobayashi, S., Nakajima, I., Yoshikawa, M., 1999. Expression of soybean  $\beta$ -1, 3-endoglucanase cDNA and effect on disease tolerance in kiwifruit plants. *Plant Cell Reports* 18, 527–532.
- Nishiyama, I., 2007. Fruits of the *Actinidia* genus. *Advances in Food and Nutrition Research* 52, 293–324.
- Nishiyama, I., Yamashita, Y., Yamanaka, M., Shimohashi, A., Fukuda, T., Oota, T., 2004. Varietal difference in vitamin C content in the fruit of kiwifruit and other *Actinidia* species. *Journal of Agricultural and Food Chemistry* 52, 5472–5475.
- Ohta, M., Hayashi, Y., Nakashima, A., Hamada, A., Tanaka, A., Nakamura, T., Hayakawa, T., 2002. Introduction of a  $\text{Na}^+/\text{H}^+$  antiporter gene from *Atriplex gmelini* confers salt tolerance to rice. *FEBS Letters* 532, 279–282.
- Ozawa, K., 2009. Establishment of a high efficiency *Agrobacterium*-mediated transformation system of rice (*Oryza sativa* L.). *Plant Science* 176, 522–527.
- Ratner, A., Jacoby, B., 1976. Effect of  $\text{K}^+$ , its counter anion, and pH on sodium efflux from barley root tips. *Journal of Experimental Botany* 27, 843–852.
- Subhasree, B., Baskar, Laxmi, K.R., Lijina, S.R., Rajasekaran, P., 2009. Evaluation of antioxidant potential in selected green leafy vegetables. *Food Chemistry* 115, 1213–1220.
- Takahashi, R., Liu, S.K., Takano, T., 2009. Isolation and characterization of plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter genes from salt-sensitive and salt-tolerant reed plants. *Journal of Plant Physiology* 166, 301–309.
- Tan, L.L., Hu, Z.H., Cai, X., Chen, Y., Shi, W.J., 2007. Histochemical localization and the content compare of main medicine components of vegetative organs in Bupleurum Chinese DC. *Journal of Molecular Cell Biology* 40, 214–222.
- Tang, R., Li, C., Xu, K., Du, Y., Xia, T., 2009. Isolation, functional characterization, and expression pattern of a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter gene *TrNHX1* from *Trifolium repens* L. *Plant Molecular Biology Reporter* 28, 102–111.
- Tian, N., Xu, Z.Q., He, J.G., 2007. Establishment of high frequency and direct regeneration system of kiwifruit (*Actinidia deliciosa* Qinmei). *Journal of Wuhan Botanical Research* 25, 79–83.
- Webby, R.F., Wilson, R.D., Ferguson, A.R., 1994. Leaf flavonoids of *Actinidia*. *Biochemical Systematics and Ecology* 22, 277–286.
- Wilkosz, R., Schläppi, M., 2000. A gene expression screen identifies *EARLII* as a novel vernalization-responsive gene in *Arabidopsis thaliana*. *Plant Molecular Biology* 44, 777–787.
- Wu, C.A., Yang, G.D., Meng, Q.W., Zheng, C.C., 2004. The cotton *GhNHX1* gene encoding a novel putative tonoplast  $\text{Na}^+/\text{H}^+$  antiporter plays an important role in salt stress. *Plant & Cell Physiology* 45, 600–607.
- Xia, T., Apse, M.P., Aharon, G.S., Blumwald, E., 2002. Identification and characterization of a NaCl-inducible vacuolar  $\text{Na}^+/\text{H}^+$  antiporter in *Beta vulgaris*. *Physiologia Plantarum* 116, 206–212.
- Xu, K., Hong, P., Luo, L.J., 2009. Overexpression of *AtNHX1*, a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter from *Arabidopsis thaliana*, in *Petunia hybrida*, enhances salt and drought tolerance. *Journal of Plant Biology* 52, 453–461.
- Xue, Z.Y., Zhi, D.Y., Xue, G.P., Zhang, H., Zhao, Y.X., Xia, G.M., 2004. Enhanced salt tolerance of transgenic wheat (*Triticum aestivum* L.) expressing a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf  $\text{Na}^+$ . *Plant Science* 167, 849–859.
- Yamaguchi, T., Blumwald, E., 2005. Developing salt-tolerant crop plants: challenges and opportunities. *Trends in Plant Science* 10, 615–620.
- Zhang, H.X., Blumwald, E., 2001. Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nature Biotechnology* 19, 765–768.
- Zhang, H.X., Hodson, J.N., Williams, J.P., Blumwald, E., 2001. Engineering salt tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. *Proceedings of the National Academy of Sciences of the United States of America* 98, 12832–12836.
- Zhu, J.K., 2003. Regulation of ion homeostasis under salt stress. *Current Opinion in Plant Biology* 6, 441–445.