TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2018) 42: 150-158

doi:10.3906/bot-1705-23

Phenotypic evaluation of transgenic peas (*Pisum sativum* L.) harboring *AtNHX1* demonstrates stable gene expression and conserved morphology in subsequent generations

Zahid ALI[1,*], Wajeeha SAEED[1], Saadia NASEEM[1], Faheem AHMAD[1], Ahmed AKREM[2], Nighat YASMEEN[1], Hans Joerg JACOBSEN[3]

[1]Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan
[2]Botany Division, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan, Pakistan
[3]Institute of Plant Genetics, Wilhelm Leibniz University of Hanover, Hanover, Germany

Received: 11.05.2017 • **Accepted/Published Online:** 06.10.2017 • **Final Version:** 20.03.2018

Abstract: In recent years, transgenic approaches have played a significant role in improving traits that help plants overcome abiotic stresses. Combined gene discovery and functional genomics have helped identify diversified mechanisms and gene families, which improved productivity under various abiotic stresses. We report on the genetic stability and persistent morphological features of transgenic pea (*Pisum sativum* L.) plants harboring the dicistronic vector construct pG0229MAS*nhx1/luc* over five generations. In addition to salt stress tolerance, the transgenic plants showed frost tolerance compared to wild-type (WT) plants. Frost tolerance of *AtNHX1* transgenic pea plants was unexpected and needs further investigation. We report morphological and molecular characteristics of transformed plants after long-term storage at 30–50 °C. The transgenic plants were morphologically stable and genetic stability of integrated genes was confirmed prior to and after transfer of plants to a glasshouse. Leaf size, shape, and color, plant height, number of tendrils, flower shape, pod shape, and grains were morphologically similar to the WT counterpart in all transgenic generations. This is the first report showing the genetic stability of transgenic pea plants harboring the salt stress tolerance gene (*AtNHX*1) from *Arabidopsis thaliana* in subsequent generations over a period of 6 years.

Key words: Transgenic pea, Na^+/H^+ antiporter, morphology, genetic stability, stress tolerance

1. Introduction

The insertion of specific traits into plants through transgenic processes for improved physiological as well as morphological parameters has become a common practice over the last 20 years. Development of genetically modified (GM) crops has become a growing industry worldwide. More than 114 million ha were under GM crop cultivation globally, and in just 3 years the area increased to nearly 150 million ha (Godfray et al., 2010; Kohli et al., 2010). Most currently cultivated GM crops harbor important traits against biotic and abiotic stress (Innes, 2008; James, 2014). The most common of these include resistance to pests and pathogens and tolerance to salinity, thermal, or moisture stresses (Ali et al., 2010). Genetic modifications are typically associated with developing a desirable, beneficial phenotype (El-Banna et al., 2010). In addition to improved food crop traits, GM plants have also gained importance for biopharmaceutical production as well as industrial products (Jefferson-Moore and Traxler, 2005; Sakakibara and Saito, 2006; Stewart and McLean, 2008). For physiological traits transformed into a specific plant against a specific stress (biotic or abiotic), it is essential to have genetic and phenotypic stability over several generations.

Since the introduction of transgenic crops, the area under cultivation of GM crops has increased every year since 1996. There was approximately an 80% increase in the area of transgenic crop production in 2009 compared to 2006 (Innes, 2008; James, 2010, 2014). Different transgenic crops are being cultivated in major agricultural producers, including both developed and developing countries. In total commodity production, the highest share of transgenic varieties is in soybean, which contribute about 77% to total soybean production, followed by cotton (49%), maize (26%), and canola (21%) (James, 2012). The US plants the largest area of transgenic crops, where about half of the area cultivated (48%) is with GMOs; this is followed by Brazil at 16%, Argentina at 15%, India at 6.3%, Canada at 6.1%, and China at 2.8% (James, 2012).

* Correspondence: zahidali@comsats.edu.pk

Pea (*Pisum sativum* L.) is an important food crop belonging to the family *Fabaceae*. It is the third most important legume after soybean and common bean (Popova et al., 2009; Murtaza and Asghar, 2013). Peas are cultivated in an area of 528 thousand ha in a variety of countries (Khan et al., 2008, 2013). Peas are a cold season crop (Bakht et al., 2009; Gopinath et al., 2009) with a short growing season (Ashraf et al., 2011). Legumes represent the second most important family of crop plants, with approximately 27% of the world's total crop production (Graham, 2003). Dry peas are the second most widely grown legume in the world, with primary production in temperate regions and global production of 10.4 Mt (http://faostat3faoorg/faostat-gateway/go/to/download/Q/QC/S). Pea cultivation in Pakistan occurs on 15,800 ha to produce 105,000 t (Attari and Javed, 2013). However, average green pod yield per unit area of the crop in Pakistan is quite low compared to other countries. The present study deals with the functional characterization of different morphological features of salt-stress–tolerant transgenic pea plants developed in our previous studies using *Agrobacterium*-mediated transformation (Ali et al., 2015). The genetic and morphological stability of transgenic peas was investigated over a period of 6 years and in five subsequent generations maintained under glasshouse conditions.

2. Materials and methods

The development of transgenic pea plants through *Agrobacterium*-mediated transformation of a cultivar provided by Prof Hans Joerg Jacobsen was described earlier (Ali et al., 2015). Subsequent generations of transgenic pea plants (line 12-07) were characterized by PCR using *AtNHX*1 specific primers At-nhx f 138 5′-ATAGATGGATGAACGAAT-3′ and At-nhx r 437 5′ GTCAAAGGTTCCAATGT-3′ (Ali et al., 2015). These plants were evaluated for salt and cold stress tolerance in this study.

2.1. Morphological characterization

Morphological characterization of transgenic pea plants was based on both qualitative and quantitative measures of flower shape, flower color, leaf color, pod color, leaf area, plant height, the number of leaves, the number of tendrils, and number of seeds per pod in subsequent generations. For comparative studies, we selected five generations of transgenic pea plants from line 12-07. Five plants of each transgenic generation as well as wild-type (WT) pea were selected to evaluate morphological characteristics. The time from the day of emergence until full maturity of the plants was recorded. Mean values of each morphological parameter of every plant was reported for each generation. The data were analyzed using the Genstat statistical package (VSN International, Ltd., Hemel Hempstead, UK) and the difference between the transgenic and nontransgenic plants was compared at probability ($P = 0.05$).

2.2. Molecular characterization

The molecular identity of each plant was confirmed by extraction and characterization of the t-DNA cassette in genomic DNA as described by Ali et al. (2010). DNA pellets were dissolved in TE buffer and treated with RNase enzyme (Fermentas; Thermo Fisher Scientific, Waltham, MA, USA) to remove RNA contamination. The amount of genomic DNA was measured using the nanodrop method. DNA was amplified in a final volume of 25 µL containing approximately 100 ng of template DNA, with 2 pM of each primer (forward and reverse), 10 mM of the dNTPs mix, 2 µL of 10X Taq DNA Polymerase Buffer (Biobasic, Markham, ON, Canada) 2 mM (final concentration) of $MgCl_2$, and 0.5 µL Taq DNA polymerase. The PCR amplification was performed in a programmed thermal cycler using a Gene Amp PCR system 2400 (PerkinElmer, Foster City, CA, USA). For *AtNHX*1 confirmation in transgenic plants, specific primers At-nhx (f)138 5′ATAGATGGATGAACGAAT3′ and At-nhx (r)437 5′AGTCAAAGGTTCCAATGT3′ were used (Ali et al., 2010; 2015). For the confirmation of *BAR*, specific primers bar(f) 5′AGCCCGATGACAGCGACCAC3′ and bar(r) 5′GCAGGAACCCGAGTGGA3′ were used. For *LUC*, specificprimersluc(f)5′CCTTCCGCATAGAACTGCCT3′ and luc(r) 5′TCCAAAACAACAACGGCG3′ were used (Ali et al., 2010). The samples were initially denatured at 94 °C for 5 min, followed by 28 cycles at 94 °C (denaturation), 45 s at the specified annealing temperature (57 °C) for each gene fragment, and 1 min extension at 72 °C. The final extension was done at 72 °C for 7 min. The amplified products were resolved in an agarose gel (1.2% w/v), visualized after staining with ethidium bromide under a UV light. Results were documented using a Biometra Gel Doc EZ Imager (Biometra, Göttingen, Germany).

2.3. Protein extraction and SDS-PAGE analysis

Proteins were extracted from fresh leaves of transgenic and WT pea plants using the trichloroacetic acid (TCA)-acetone precipitation method with some modifications. Leaves and seeds were crushed to a fine powder in a pestle and mortar with liquid nitrogen. The powder was transferred into Eppendorf tubes and extraction buffer (0.5 M Tris-HCl (pH 8.0, 10% SDS, Urea, 1.5% β-mercaptoethanol)) was added. Precipitation of proteins was done using TCA with 0.07% β-mercaptoethanol dissolved in acetone. Extracted protein concentration was performed using a Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) used as a standard. Six standards were prepared (S1, S2, S3, S4, S5, and S6) containing 0, 2.5, 5, 10, 15, and 20 µL of BSA stock (1 mg/mL), respectively, in 1 mL of Bradford

reagent. In addition to that, 5 µL of each protein sample was mixed with 1 mL of Bradford reagent. Absorbance was measured using a nanodrop at 595 nm in a cuvette. The BSA standard curve was used to estimate the concentrations of protein samples. The extracted proteins from transformed and WT control plants were separated using denaturing protein electrophoresis. Proteins were stained using 0.1% Coomassie R250 followed by destaining (20% ethanol, 10% acetic acid, 70% distilled water) and visualization under UV light.

2.4. Frost acclimation and chlorophyll content analysis

The study was conducted from mid-November 2013 to the end of January 2014. The ambient temperature at the research facility at CIIT in Islamabad at the time of sowing was approximately 25 °C. Seeds of 25 transgenic (F_1 progeny) and 25 WT plants were soaked in water one day before sowing. Seeds were grown in 30% sand and 70% soil in 1-L pots. Seeds emerged 1.5–2 weeks after sowing. Freezing temperatures of –1 °C to –2 °C occurred at the end of December with a maximum of –3 °C (night temperature) recorded from the Pakistan Weather Portal. Five leaves of each plant were taken to measure the chlorophyll content using a SPAD 502 Plus Chlorophyll Meter (SPAD-502, Konica-Minolta, Tokyo, Japan) for nondestructive, rapid measurement of leaf chlorophyll using the calibration curve method described by Ling et al. (2010).

3. Results

3.1. Plant morphology

The plants of multiple generations of transgenic pea (line 12-07), developed from the seeds maintained over a period of 6 years, showed morphological and phenotypic stability in five subsequent generations. No differences were observed in flower color or shape of transgenic plants compared to nontransgenic plants with or without 100 mM NaCl stress. The flower color was white for both, and the transgenic and WT plants had the same flower shape (Figure 1A). Additionally, the leaves of the transgenic lines and the veins were clear and slightly whitish compared to the WT. However, the color of leaves was green in both transgenic and WT plants (Figure 1B). The pod color was green in transgenic plants as well as the WT, and the shape of pods was blunt and dehiscent in transgenic and WT plants. The shape and color of the pod and grain remained the same in both types under normal/stressed conditions (Figure 1C). Plant height was also similar for both transgenic and WT pea plants (Figure 1D).

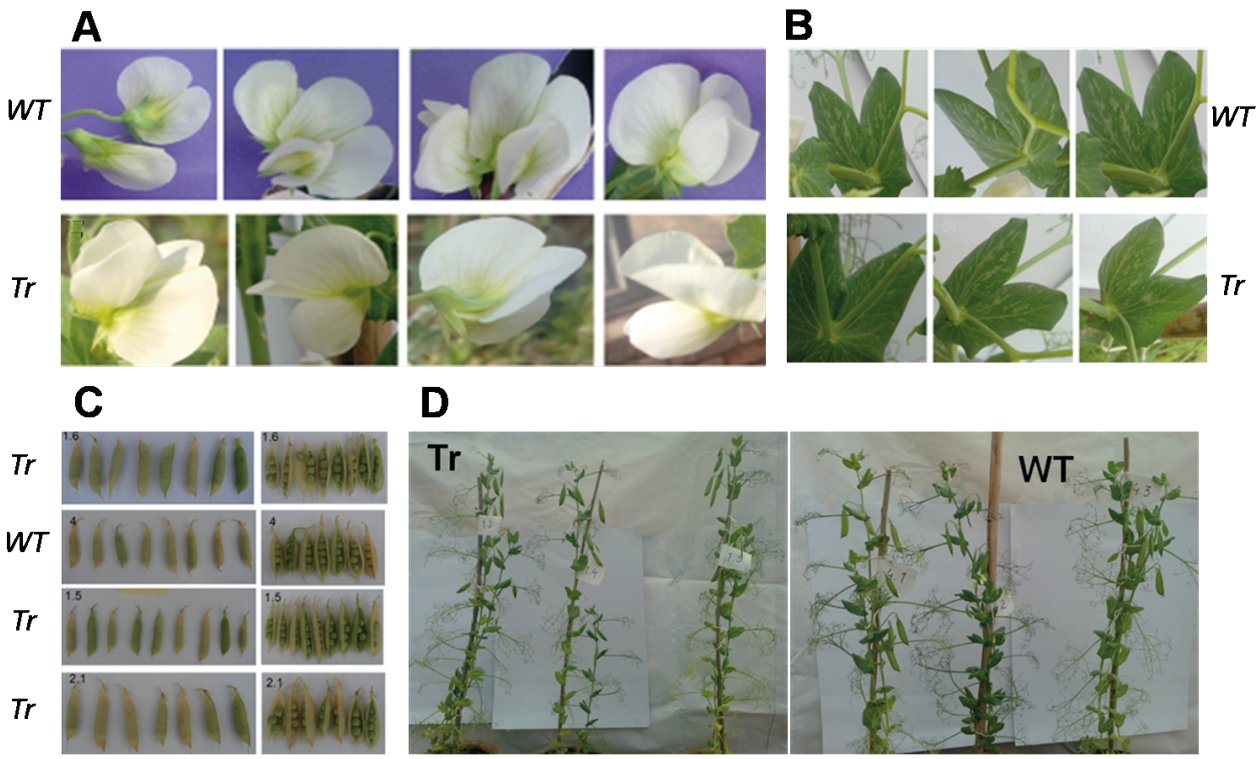

Figure 1. Comparative morphological features of transgenic and WT pea plants: A) flower morphology, B) leaf shape and color, C) shape and color of pod and grain, D) plant height. There was no difference in morphological features of transgenic and WT plants in subsequent generations.

There was variation in the number of leaves (Figure 2A), internodal distance (Figure 2B), plant height (Figure 2C), and number of tendrils (Figure 2D) in transgenic and WT plants. However, no significant difference was observed in the morphological features of transgenic or WT pea plants (Figure 2A–2D).

3.2. Leaf area and root growth under salt stress

Transgenic plants showed an increased leaf area and better root growth performance under salt stress. Leaf area is an important parameter to measure plant growth under stressful conditions, including salt stress. The results showed a significant decrease in leaf area of WT pea plants under NaCl stress compared to unstressed plants (Figure 3). However, no significant difference was observed in the leaf area of transgenic pea plants under salt stress in comparison to unstressed plants (Figure 3). The better root growth performance of transgenic plants in comparison to the WT counterpart under 100 mM NaCl stress has already been shown (Ali et al., 2015).

3.3. Molecular characterization

Molecular characterization of transgenic pea plants was done by PCR analysis. PCR analysis of genomic DNA from leaves and seeds of transformed plants grown in the glasshouse demonstrated the presence of all genes including *AtNHX*1, *BAR*, and *LUC* (Figure 4). Hence, none of the T-DNA genes was lost over a period of 6 years of maintenance in controlled glasshouse conditions. All the PCR products were of an expected size and were identical to those of the positive control. For protein analysis, there was a difference in protein profiling of transgenic and WT plants (Figure 5).

3.4. Frost tolerance

Time of flowering was delayed for the WT plants for about 3 weeks due to frost and low temperature, although transgenic plants flowered normally (Figures 6A and 6B). Chlorophyll content analysis of transgenic pea plants against frost stress showed remarkable compliance to this notion (Figure 7).

4. Discussion

Genetic engineering has provided a consistent increase in the global area planted with GM crop plants since 1990. For this purpose, transgenic plants for variable traits have been developed, starting with insect-resistant maize, cotton, and potato; herbicide-tolerant soybean, maize, cotton, canola, and rice; and virus-resistant squash and papaya, which were planted in different regions of the

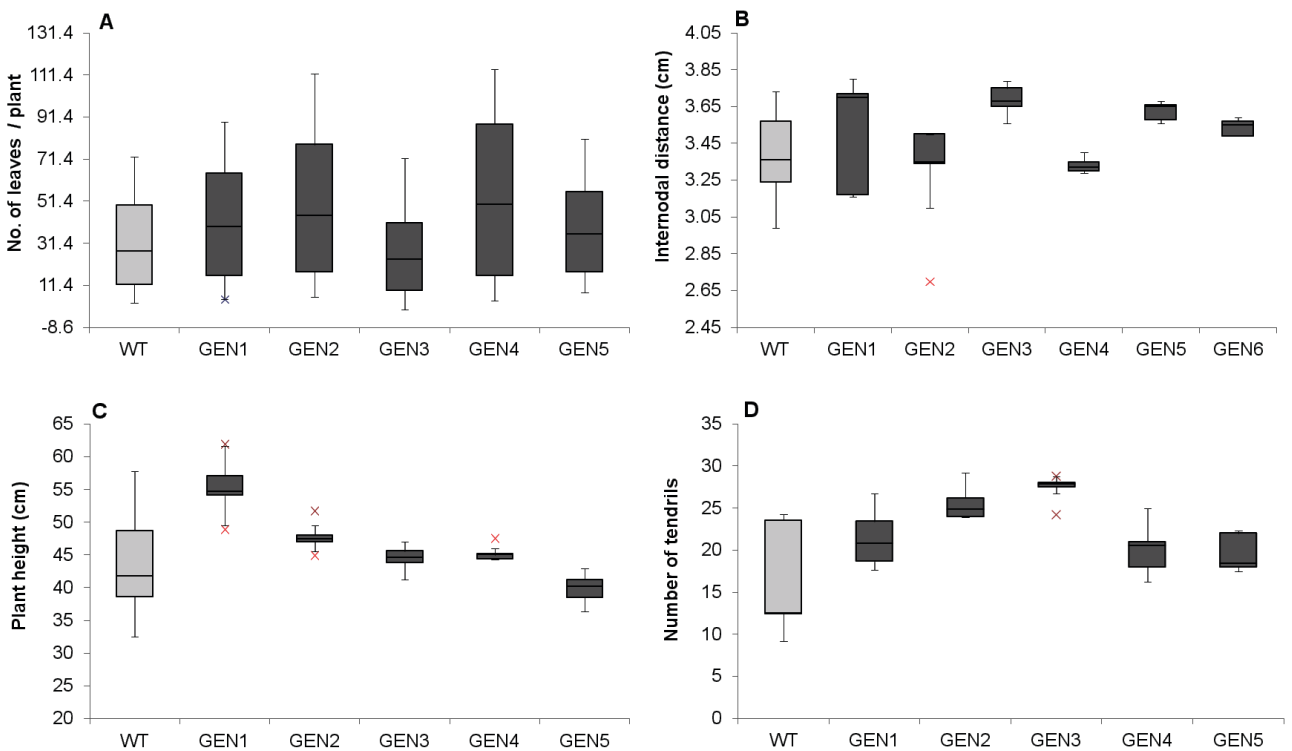

Figure 2. Comparative data on morphological characteristics of transgenic and WT pea plants. A) Number of leaves, B) internodal distance, C) plant height (cm), D) number of tendrils. The data from each generation (GEN) of transgenic and WT plants are averages of 5 plants.

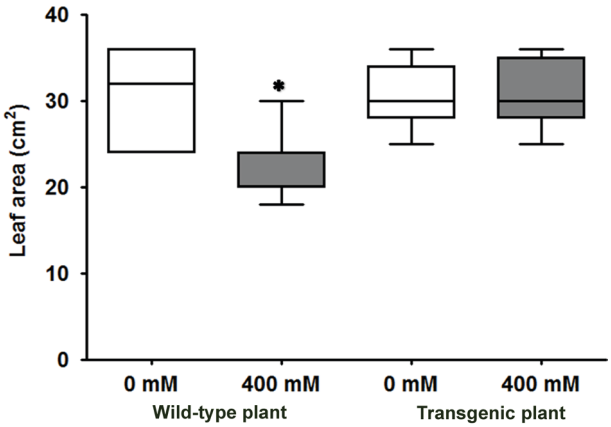

Figure 3. The difference in leaf area of transgenic and WT pea plants under salt stress. WT plants showed a significant decrease in leaf area in comparison to transgenic plants under salt stress when treated with 400 mM NaCl solution.

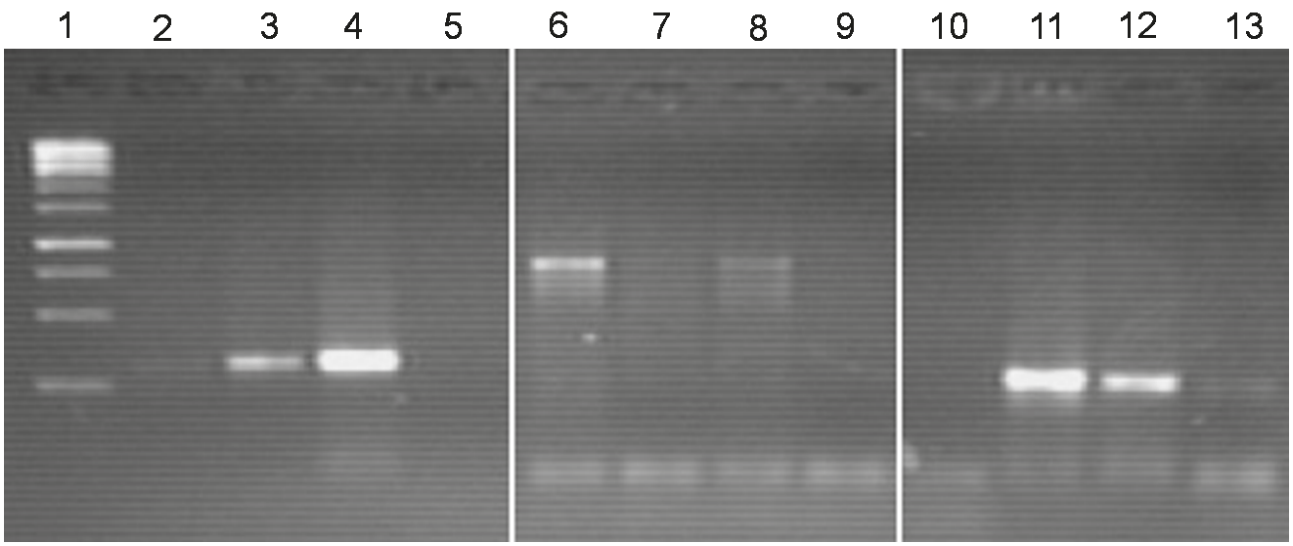

Figure 4. PCR confirmation of transgenic pea plants (generation 5 of line 12-06). Lanes 2–4 show amplification of *AtNHX*1 using specific primers, Lanes 7 and 8 represent amplification of *LUC* using 837 bp specific primers, Lanes 11–13 show amplification of *BAR* using 232 bp specific primers and Lanes 5, 9, and 10 are for WT pea plants, while Lane 1 is 1 Kbp DNA marker.

world with striking market value (James, 2012). With advanced genetic engineering tools, more than one trait has been incorporated or stacked into transgenic plants, which resulted in crops with combined traits (insect and herbicide tolerance) in transgenic maize and cotton, which are available commercially (James, 2012). Although different GM plants have been developed successfully against different biotic and abiotic stresses, sometimes the plants may display a very typical phenotype due to the transgene that differs from their nontransgenic counterparts (Casanova et al., 2005). Such characteristics include wrinkled leaves, reduced apical dominance, improved flower morphology, increase in the number of branches, decreased seed and pollen production, and ample production of highly branched plagiotropic roots (Casanova et al., 2005). Besides these mentioned changes, due to transformation with *A. rhizogenes*, some species that grow biennially, e.g., carrot, switched to an annual cycle due to rolC, which was the primary promoter of annualism (Tepfer, 1984; Limami et al., 1998).

Development of transgenic legume plants is an important area of research and these legumes are widely used as food and fodder. Nevertheless, for establishing the integrity of such new transgenic plant varieties, studies of different

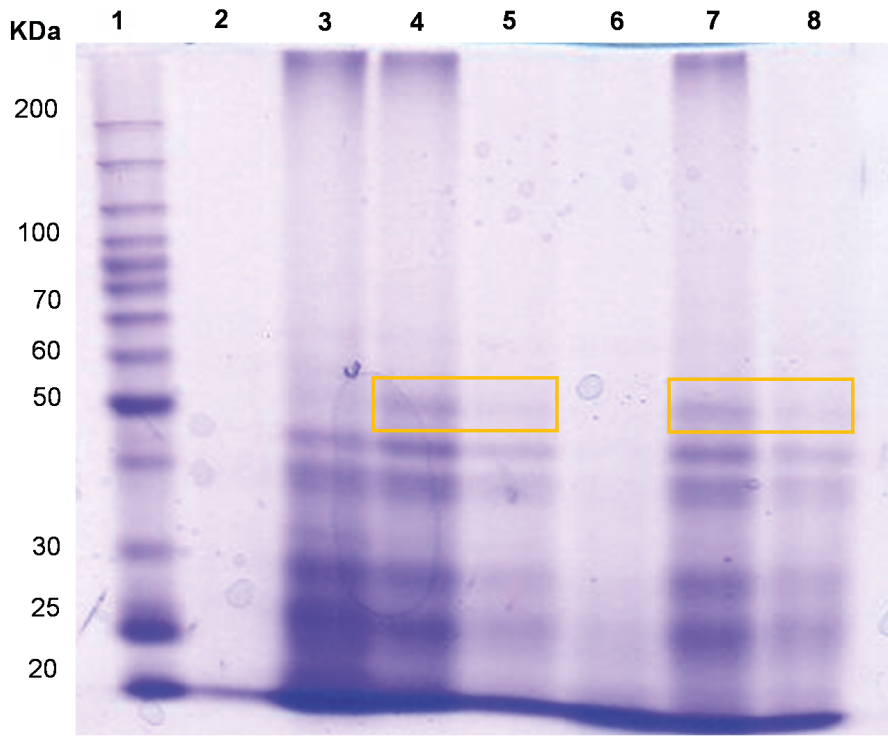

Figure 5. Protein profiles of transgenic and WT pea plant using SDS-PAGE. Lane 1 is the standard protein ladder with their corresponding molecular weights, Lane 3 was loaded with 0.5 µg of WT plant protein. Lanes 4, 5, 7, and 8 show the complete protein banding pattern of transgenic plant. Lanes 4 and 7 were loaded with 0.5 µg and lanes 5 and 8 were loaded with 0.25 µg of extracted proteins, respectively. Yellow square highlights the persistent presence of AtNHX1 protein (approximately 50 KDa) in transgenic leaves.

Figure 6. The flowering of transgenic (A) and WT (B) pea plants, 60 days postgermination. Transgenic plants flowered on time, while flowering of WT plants was delayed by 3 weeks under frost stress.

generations are necessary. The present research focuses on genetic stability and persistence of morphological features of transgenic pea plants harboring a Na^+/H^+ antiporter from *A. thaliana* gene, which is a known trait against salt stress (Apse et al., 2003). These transgenic plants harbor a dicistronic expression cassette *AtNHX*1 and *LUC* (driven

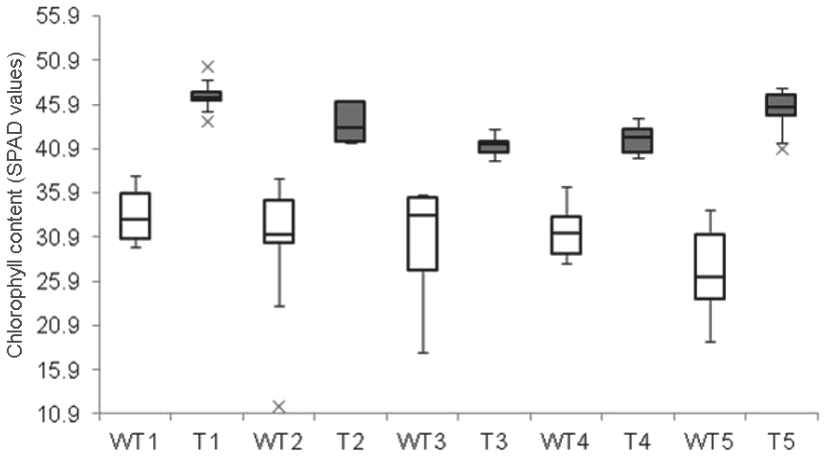

Figure 7. Chlorophyll contents (SPAD values) of WT (white box) and transgenic (T) pea plants (gray box). Five plants from each transgenic generation and WT plants were selected to observe the frost tolerance potential. Chlorophyll contents were determined from five healthy green leaves from each plant in triplicate.

by manopine synthase promoter) in opposite orientation to the *BAR* gene (driven by Nos promoter) (Ali et al., 2010). The transgenic plants were morphologically more stable under salt-stressed conditions in the subsequent generation of the transgenic plant, as they retain the transgenic characters typical for the *AtNHX*1-transformed plants (Christey, 1997). The combined *AtNHX*1 and *LUC* expression (driven by manopine synthase promoter) and *BAR* gene expression (driven by independent promoter) may result in altered characters of transformed plants. The *AtNHX*1 gene is responsible for salinity tolerance, which is associated with greater than normal levels of AtNHX1 expression protein and vacuolar Na^+/H^+ antiporter activity (Apse, 1999). Moreover, the *AtNHX*1 from *A. thaliana* is the richest antiporter, which is involved in maintaining cellular pH and homoeostasis (Sottosanto et al., 2007). After transformation of the gene of interest to any target cell or tissue and its development to viable whole plant, the main challenge is always genetic stability. GM kiwi plants have shown stability for about 6 years (Rugini et al., 1997); however, genetic instability has also been seen in some Ri-transformed plants after a longer time. *Solanum tuberosum* is one example of spontaneous deletion of TL and TR DNA during long-term cultures (Cate et al., 1990). In the present research, the *AtNHX*1-transformed *P. sativum* plants were effectively adapted to salt-stressed conditions. Although several other plants have been developed using Na^+/H^+ antiporter gene like Arabidopsis (Apse, 1999), tomato (Zhang and Blumwald, 2001), buckwheat (Chen et al., 2007), and cotton (He, 2005), the genetic stability of transgene and persistence in morphological features in subsequent generations have not been explored. This is the first report of the successful adaptation of *AtNHX*1-transformed *P. sativum* plants to soil in controlled conditions. The plants exhibited preservation of most of the transgenic morphological features, and retained and expressed the *AtNHX*1 genes, indicating genetic stability of the *AtNHX*1-transformed plants in five subsequent generations continuously growing in the soil pots under controlled conditions. However, there were variations among the number of leaves, flower, branches, pods, etc. This variation was observed between both transgenic and WT plants. We could not observe any structural differences in morphological features and any color variation in leaves, flowers, pods, grains etc., as well as life cycle of transgenic and WT plants. Studies have shown that after transfer of transgenic plants to glasshouses, the modified characteristics were conserved. Some examples are *Datura arborea* (Giovannini et al., 1997) and *Kalanchoe blossfeldiana* (Christensen et al., 2009). The *AtNHX*1-transformed *P. sativum* also exhibited stability in plant height and better root growth performance under NaCl stress conditions; however, WT plants showed reduced leaf area and root growth (Figure 3). Some clones of *D. arborea* displayed equal plant height to the control after 6 months in the greenhouse (Giovannini et al., 1997). In the present research, the *AtNHX*1-transformed *P. sativum* plants showed better tolerance against salt stress and genetic stability in subsequent generations and exhibited persistence in morphological characters. Various investigations of genetic and molecular characteristics of transgenic plants have mentioned a significant interaction among thermal and osmotic stresses and ABA responses (Xiong, 1999). Mild stress of one type can substantially increase tolerance level of plants to multiple stresses (Tamirisa et al., 2014). Time of flowering of pea plants,

which depends on photoperiod and temperature and varies from 60 to 70 days after sowing in the local climatic conditions of Pakistan, was delayed for the WT counterparts for about 3 weeks due to frost and low temperature, although transgenic plants flowered normally as shown in Figures 6A and 6B). Chlorophyll content analysis of transgenic pea leaves showed remarkable compliance with this notion. The result indicated that transgenic pea plants showed better chlorophyll contents under freezing stress and timely flowering than WT (Figures 6A and 6B). The results were in consensus with previous studies on different plants, in which they confirmed the increased freezing tolerance related to ABA accumulation upon exposure to mild drought stress (Guy et al., 1985; Mantyla et al., 1995; Xiong, 1999; Tamirisa et al., 2014). The molecular basis of overlapping stress responses involving ABA as a key regulator of dual stress responses in *Arabidopsis* mutants has also been well documented (Ishitani et al., 1997). SPAD values show chlorophyll content of both transgenic and WT pea plants (Figure 7). Nevertheless, further investigations of increased tolerance at low temperature by the transgene *AtNHX*1 may reveal new avenues of multiple stress adaptation by this gene and the possible role of ABA and AtNHX1 interaction in modulating stress responses in transgenic pea plants. Moreover, our transgenic seeds show promising results regarding reproductive development where the yield characteristics including flowering, number of pods, and seeds per pod, and yield were comparable to nontransgenic counterparts. Our results are consistent with earlier studies (Tuteja et al., 2014). To our knowledge, this is the first report that shows the genetic stability of transgenic pea plants harboring *AtNHX*1 responsible for salt stress tolerance in subsequent generations over a period of 6 years.

Acknowledgments

This study was partially supported by the Higher Education Commission of Pakistan under the SRGP program. The utilization of internal resources of CIIT Islamabad is highly acknowledged.

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