

# Enhanced tolerance to drought and salt stresses in transgenic faba bean (*Vicia faba* L.) plants by heterologous expression of the *PR10a* gene from potato

Moemen S. Hanafy · Antar El-Banna ·  
Heinz Martin Schumacher · Hans-Jörg Jacobsen ·  
Fathi S. Hassan

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## Abstract

**Key message** We report for the first time that expression of potato *PR10a* gene in faba bean causes enhanced tolerance to drought and salinity.

**Abstract** Grain legumes such as soybean (*Glycine max* L. Merrill), pea (*Pisum sativum* L.) and faba bean (*Vicia faba* L.) are staple sources of protein for human and animal nutrition. Among grain legumes, faba bean is particularly sensitive to abiotic stress (in particular osmotic stress due to lack of water or enhanced soil salinity) and often suffers from severe yield losses. Many stress responsive genes have been reported with an effect on improving stress tolerance in model plants. Pathogenesis-related proteins are

expressed by all plants in response to pathogen infection and, in many cases, in response to abiotic stresses as well. The *PR10a* gene isolated from the potato cultivar Desiree was selected for this study due to its role in enhancing salt and/or drought tolerance in potato, and transferred into faba bean cultivar *Tattoo* by *Agrobacterium tumefaciens*-mediated transformation system based upon direct shoot regeneration after transformation of meristematic cells derived from embryo axes. The transgene was under the control of the constitutive mannopine synthase promoter (p-MAS) in a dicistronic binary vector, which also contained luciferase (*Luc*) gene as scorable marker linked by internal ribosome entry site elements. Fertile transgenic faba bean plants were recovered. Inheritance and expression of the foreign genes were demonstrated by PCR, RT-PCR, Southern blot and monitoring of Luciferase activity. Under drought condition, after withholding water for 3 weeks, the leaves of transgenic plants were still green, while non-transgenic plants (WT) wilted and turned brown. Twenty-four hours after re-watering, the leaves of transgenic plants remained green, while WT plants did not recover. Moreover, the transgenic lines displayed higher tolerance to NaCl stress. Our results suggested that introducing a novel *PR10a* gene into faba bean could be a promising approach to improve its drought and salt tolerance ability, and that MAS promoter is not only constitutive, but also wound-, auxin/cytokinin- as well as stress-inducible.

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M. S. Hanafy · H.-J. Jacobsen · F. S. Hassan (✉)  
Plant Biotechnology Department, Leibniz University Hannover,  
Institute of Plant Genetics, Herrenhäuser Str. 2,  
30419 Hannover, Germany  
e-mail: hassan@lgm.uni-hannover.de

M. S. Hanafy  
Plant Biotechnology Department, National Research Centre  
(NRC), Tahrir Str. Dokki, 12311 Cairo, Egypt

## Present Address:

M. S. Hanafy  
Faculty of Science and Humanities, Biology Department,  
Salman bin Abdulaziz University (SAU),  
11942 Al-Kharj, Saudi Arabia

A. El-Banna  
Faculty of Agriculture, Department of Genetics, Kafr  
El-Sheikh University, 33516 Kafr El-Sheikh, Egypt

H. M. Schumacher  
German Collection of Microorganisms and Cell Cultures DSMZ  
GmbH, Inhoffenstr. 7b, 38124 Braunschweig, Germany

**Keywords** *Vicia faba* · *Agrobacterium*-mediated transformation · Abiotic stress · *PR10a*, IRES elements · Heterologous expression

## Abbreviations

*bar* Bialaphos resistance gene

PPT	Phosphinothricin
BAP	6-Benzylaminopurine
IBA	Indole-3-butyric acid
CTAB	Cetyltrimethylammoniumbromide
PR	Pathogenesis-related

## Introduction

Protein-rich grain legumes (pulses) are globally important crops; regions of production include drought-prone areas. However, environmental stress factors impose major limitations on pulse productivity. Abiotic stresses such as drought and salinity can reduce grain yields dramatically. Plants react to changes in their environment by large-scale regulation of their metabolism in order to cope with these challenges including stress-avoidance strategies (early maturing, adaptive growth habit and morphological changes). The metabolic changes are initiated by a differential expression of a subset of genes responding either specifically to a certain stress or unselectively to different stresses (Shinozaki et al. 2003). Identification of genes involved in tolerance to drought and/or salinity and transfer of these genes into commercial cultivars either through breeding or through genetic transformation could therefore significantly increase the agronomic potential of legumes and will thus contribute to improve food security for an expanding population mainly in developing countries (Pellegrineschi et al. 2005). Complex characters, such as abiotic stress tolerance, are difficult to select for and are scarce in the narrow gene pools of pulses such as faba bean (Bond 1987). One promising approach therefore is to integrate useful drought/salt-tolerant traits through genetic engineering of susceptible species. Several genes carrying these traits have been identified (Shinozaki and Yamaguchi-Shinozaki 2000; Xiong and Zhu 2002; Tran et al. 2004; Ma et al. 2006; OuYang et al. 2007; El-Banna et al. 2010; Xianjun et al. 2011). Applied together with traditional breeding, these genes may offer sustainable solutions for farming in water-limited conditions. The engineering of drought-tolerant crop plants has been a long-held and intensively sought objective. A wide range of genes encoding different structural and regulatory proteins have been employed over the past years, for developing abiotic stress-tolerant transgenic plants (see review, Bhatnagar-Mathur et al. 2008).

The *Pathogenesis-related* (PR) proteins comprise a large super-family of plant defense proteins that accumulate in response to various biotic and often also abiotic stresses (Liu et al. 2003; Srivastava et al. 2004; El-Banna et al. 2010). The major families of PR proteins have been grouped at least into 17 different classes, primarily based on their amino acid

sequence similarity (Van Loon and Van Strien 1999). The PR10 proteins are elicitor- and osmotically inducible proteins, which exhibit ribonuclease activity (Somssich et al. 1986). The group of PR10 proteins was defined by Van Loon et al. (1994). This group included the PR10a protein (Marineau et al. 1987) from potato. The biotic and abiotic stress-inducible nature of the *PR10* genes include pathogen infection (McGee et al. 2001; Tanaka et al. 2003), salt tolerance (El-Banna et al. 2010; Jain et al. 2012), UV irradiation (Rakwal et al. 1999), osmotic tolerance (Vaas et al. 2012) and ozone stress (Agrawal et al. 2002).

The first objective of the present study was to find out whether abiotic stress tolerance can be achieved in faba bean by expression of *PR10a* gene from potato without any visible effects on plant growth and development, compared to previous work of El-Banna et al. (2010) which studied the effect of *PR10a* gene at cellular level using well-established potato cell culture. The present study, however, demonstrates the effect of *PR10a* gene at multi-cellular level using whole plants of faba bean. The second objective was to characterize the transgenic events of faba bean under abiotic (drought and salt) stress. To achieve these objectives, a dicistronic transformation vector was used (El-Banna et al. 2010). In this vector, the *PR10a* gene is linked by an internal ribosome entry site (IRES) sequence to the *Luc* reporter gene. This construct leads to the formation of a unique mRNA for both genes, but the formation of two physically independent proteins, assuring full physiological functionality of both target and reporter genes (Dorokhov et al. 2002). The *PR10a* gene expression can simply be monitored by assaying the reporter gene expression. In the present study, faba bean plants have been transformed using *Agrobacterium*-mediated transformation method which was established by Hanafy et al. (2005) using *bar* gene as a selectable marker and *PR10a* gene from potato for enhancing its tolerance to drought and salinity.

## Materials and methods

### Plant material

Mature faba bean seeds (*Vicia faba* L.) cv. 'Tattoo' were surface sterilized in 70 % (v/v) ethanol for 1 min followed by 6 % sodium hypochlorite (v/v) for 7 min followed by washing thoroughly with sterile distilled water for 3–4 times. The seeds were then soaked overnight in sterile distilled water.

### Bacterial strains and vectors

The hypervirulent *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) harboring the pGreenII 0229

derivative binary vector containing a *bar* gene and the pSoup helper plasmid (Hellens et al. 2000) was used. The binary vector pGreenII 0229 has been modified to include IRES element leading to a cap-independent manner of translation (Kieft et al. 2001; Terenin et al. 2005). This vector contains the *PR10a* gene under MAS promoter as the first cistron linked by an IRES sequence to *Luc* as the second cistron.

#### Transformation of faba bean by *Agrobacterium*

The dicistronic transformation vector pGII0229MAS-PR10a-cp148-Luc (Fig. 1) was used for transformation of faba bean. *Agrobacterium*-mediated transformation of faba bean was performed as previously described (Hanafy et al. 2005) with some minor modifications using *bar* gene as a selectable marker.

Briefly, mature embryos were dissected from surface-sterilized seeds and thinly sliced using a scalpel dipped in an *Agrobacterium tumefaciens* suspension culture. The sliced explants were incubated for 30 min in the suspension prior to being densely plated on co-cultivation medium for 72 h in the absence of selection in the dark. The explants were then placed on selection medium and transferred to fresh medium at 14- to 21-day intervals under 24 °C with a 16/8-h day/night photoperiod. Transgenic shoots were selected on medium supplemented with 2 mg/l phosphinothricin (PPT). After regenerated shoots had developed and produced more than two leaves, they were excised individually and micrografted on a non-transgenic rootstock (Pickardt et al. 1995; Hanafy et al. 2005). Individual shoots are often resistant to rooting and hence grafting is a viable alternative to establish plantlets. After substantial shoot and root growth, plants were hardened and directly transplanted to soil in a greenhouse and propagated via controlled self-fertilization. The T0 plants (original transgenic plants) and their progenies (T1 and T2 plants) were grown and allowed to produce seeds under 16/8 h day/night photoperiod and at a controlled temperature of 25 °C in the greenhouse. The presence or absence of *PR10a*, *Luc* and *bar* genes in transgenic plants of the T1

and T2 generations was determined by PCR analysis. T2 plants were analyzed for drought/salt stress tolerance.

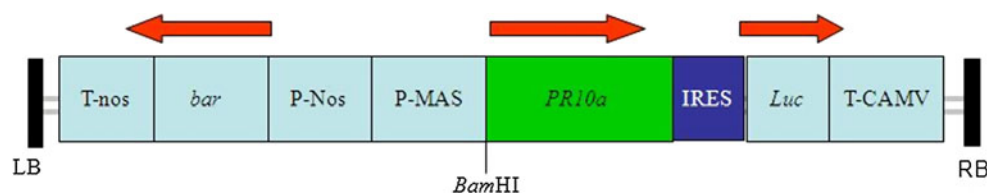
#### PCR analysis of transgenic plants

Screening for transgenic plants among T0 regenerated plants resistant to PPT and following generations (T1, T2 and T3) was performed by PCR analysis. Total DNA was extracted from the leaves of regenerated plants according to the method previously described (Sul and Korban 1996) from young leaves with minor modifications. Standard PCR protocol was used. Cycling conditions were: 94 °C, 5 min; (1 min at 94 °C, 1 min at 57 °C, 1 min at 72 °C) 30 cycles, then a final step of 10 min at 72 °C. The following primers were used:

1. PR10-For 5'-ATGGGTGTCAGCTAGCTATACACATG-3' and PR10-Rev 5'-TTAAGCGTAGACAGAAGGATTGGC-3' amplifying a 480-bp *PR10a* sequence;
2. *Luc* 834F2-5'-CCTTCCGCATAGAAGTGCCT-3' and *Luc* 834R2 5'-TCC AAA ACAACAACGGCG-3' amplifying a 834-bp *Luciferase* sequence;
3. *bar* 447 F: 5'-GATTTTCGGTGACGGGCAGGA-3'; *bar* 447 R: 5'-AACTTCCGTACCGAGCCGCA-3' amplifying a 447-bp fragment of the *bar* gene. As an internal control, primers against the *actin1* gene (accession no. J01298) were used which amplify a 831-bp fragment (*Act1*-931-For 5'-GACGCTGAGGATATTCAACC-3' and *Act1* 931-Rev 5'-AGAAATCTGTGAGGTCACGA-3').

#### Leaf paint test

The putative transgenic plants were tested in the greenhouse for the expression of *bar* gene by painting the leaflets with BASTA® (a commercial formulation of PPT containing 200 g/l ammonium glufosinate, Hoechst Ltd.) diluted to a concentration of 200–300 mg/l ammonium glufosinate. The opposite leaflet of each pair was marked and left untreated as a control. T1 and T2 generations were tested by the same method.



**Fig. 1** Schematic representation of the T-DNA region of the transformation vectors (not drawn to scale). *Tnos* 3' terminator sequence of the nopaline synthase gene, *bar* *bar* gene, *P-Nos* promoter sequence derived from nopaline synthase, *P-MAS* manopine

synthase promoter, *PR10a* pathogenesis-related proteins, formally sth2, *IRES* internal ribosome entry site, *Luc* luciferase gene, T-CAMV terminator sequence of the cauliflower mosaic virus. The right (*RB*) and left (*LB*) border sequences are represented by solid boxes

### Luciferase activity detection

Luciferase activity was monitored using a cooled, back-illuminated, charge-coupled-device (CCD) camera system. Leaves were washed with detergent, rinsed with water and dried on filter paper. A 1-mM solution of D-Luciferin potassium salt (PJK GmbH, Germany) in water was sprayed over the leaves of transgenic and WT plants. After 5 min of incubation at room temperature, the chemiluminescence was detected.

### Southern blot analysis of transformed plants

Total DNA for Southern blot analysis was isolated from young leaves of transgenic plants according to the DNeasy Kit (Qiagen, Valencia, CA). The DNA (20 µg) was digested with *Bam*HI, and the resulting fragments were fractionated by electrophoresis on a 0.8 % agarose gel (w/v) and then transferred to a positively charged nylon membrane (Roche, Germany) as described by Sambrook et al. (1989). A fragment of *PR10a* amplified from the transformation binary vector with the corresponding primer set used for PCR analysis served as a hybridization probe. The fragment was labeled and detected with the use of PCR-DIG Mix (Roche, Germany) according to manufacturer's instruction. The blots were exposed to X-ray film for 1 h.

### Isolation of total RNA and RT-PCR analysis

Total RNA was isolated from young leaves of T2 plants with the use of RNAtidy G solution (A 2867, AppliChem, Germany). A portion of the RNA (5 µg) was incubated for 30 min at 37 °C with 3 U of RNase-free DNase (MBI Fermentas, St. Leon-Rot, Germany) in 10 µl of a solution containing 10 mM Tris-HCl (pH 7.5), 2.5 mM MgCl<sub>2</sub>, and 0.1 mM CaCl<sub>2</sub>. The RNA was then subjected to phenol-chloroform extraction and ethanol precipitation before reverse transcription (RT) with an oligo (dT)<sub>20</sub> primer and a RevertAid<sup>TM</sup> H Minus First-Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). The resulting cDNA was subjected to PCR with *PR10a* primer and *actin1* primer as internal control. The PCR conditions were the same as those used for PCR analysis of genomic DNA.

### Stress tolerance of plants grown in soil

Different sets of experiments were executed in order to assess potential drought/salt tolerance of *PR10a*-expressing plants. Transgenic and non-transgenic (WT) faba bean seeds were germinated in soil (peatmoss or perlite) in a greenhouse (16/8 h day/night photoperiod) at 25 °C. Transgenic (T2 generation) and WT plants were grown in pots (size 6 l) and watered every 2 days for 4 weeks before performing the

drought/salt stress experiments. For drought stress, at week 5, the WT and transgenic plants were watered once and subjected to 3 weeks of drought followed by the previous watering schedule. For salt stress, at week 5, transgenic and WT plants were treated with 250 ml of 200 mM NaCl every third day for 6 weeks. All experiments were repeated at least twice with nine plants from each transgenic line and WT. The responses of the plants to stress were observed. The plants grown in perlite were removed from the substrate and the roots were soaked in 150 mM NaCl for 3 days. *PR10a* expression levels in leaves and roots of non-stressed and stressed transgenic and WT faba bean for 23 h and 3 days were studied by real-time PCR. For air drying stress, the plants were removed from the perlite and air dried for a maximum 20 h at room temperature (22 °C). Analysis of *PR10a* gene expression in leaves and roots in unstressed and stressed transgenic and WT faba bean plants for 4 and 20 h by air drying stress was performed by real-time PCR.

### Proline determination

Free L-proline was determined according to Bates et al. (1973) with minor modifications. Leaf explants of 250 mg were ground in a pre-cooled mortar under liquid nitrogen. The homogenate was re-suspended in 10 ml of 3 % aqueous sulphosalicylic acid (w/v) in a 50-ml Erlenmeyer flask. The flask was shaken for 20 min and the cell debris was removed by filtering through filter paper (Whatman plc, Maidstone, UK). For proline determination, 2 ml of the extracts were mixed with 2 ml ninhydrin reagent [ninhydrin 1.25 g, glacial acetic acid 30 ml, phosphoric acid (6 M) 20 ml] and 2 ml glacial acetic and incubated for 1 h at 100 °C.

The reaction was stopped by placing the tubes in an ice bath. The reaction mixture was extracted with 4 ml toluene and mixed vigorously for 15–20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene as a blank. Proline content was calculated from a standard curve on a fresh weight basis. The experiment was repeated twice using T2 generation with three transgenic plants from line TL-3 and WT.

### Real-time PCR analysis for the stressed plants

Quantitative real-time RT-PCR (qRT-PCR) was performed using SYBR Green PCR master mix kit (BioRad) in a Cycler CFX96<sup>TM</sup> Real-Time PCR Detection System (BioRad) using synthesized cDNA as template. For detecting expression profile of *PR10a* in WT and transgenic faba bean under stressed and unstressed conditions using qRT-PCR, 100 mg of leaves and roots were sampled and frozen in liquid nitrogen for RNA extraction and cDNA synthesis. Total RNA was extracted using the

RNATidyG (AppliChem, Germany). 5 µg of RNA was treated with DNase I (MBI Fermentas, St. Leon-Rot, Germany) and subsequently tested for the presence of DNA residues by PCR as described by Li et al. (2007) using the primers Act1-931F and Act1-931R amplifying a 831-bp of faba bean *actin* sequence if the RNA was contaminated with DNA. The remaining RNA was reverse transcribed using oligo(dT)<sub>20</sub> primers and the RevertAid<sup>TM</sup> H Minus First-Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instruction. The success of reverse transcription was verified by PCR of 1 µl cDNA using primers Act1-931F and Act1-931R as described before.

Reactions for real-time PCR were prepared in a total volume of 15 µl containing: 1.0 µl cDNA, 0.3 µl of each 10 pmol/µl primer, 7.5 µl of iQ<sup>TM</sup> SYBR<sup>®</sup> Green Supermix (Bio-Rad) and 5.9 µl RNase/DNase-free sterile water (Qiagen). Triplicate quantitative assays and blank controls were performed for each master mix. The cycle conditions were set as follows: initial template denaturation at 95 °C for 3 min, followed by 39 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 20 and 30 s elongation step at 72 °C followed by one step at 95 °C for 10 s. This cycle was followed by a melting curve analysis, ranging from 65 to 95 °C, with temperature increase by steps of 0.5 °C every 5 s. Baseline and threshold values were automatically determined for all plates using the CFX manager software 1.5 (Bio-Rad). The relative quantification method (Delta–Delta CT) was used to evaluate quantitative variation between the replicates examined.

The amplification of actin1 and the eukaryotic elongation factor 1-alpha (ELF1A) (Gutierrez et al. 2011) were used as internal control samples to normalize all data. Gene-specific primers for *PR10a* were rPR10-1F 5'-ATGGGTGTCAC TACTATAC-3' and rPR10-2R123 5'-CAACATTCTTAACAT TTGGC-3'; for *actin1* in faba bean, ACTI-120-For 5'-TATGGGCCAGAAAGATGCAT-3' and ACTI-120-Rev. 5'-CAA ATCTTTTCCATATCATCCAGT-3'. For ELF1A, the

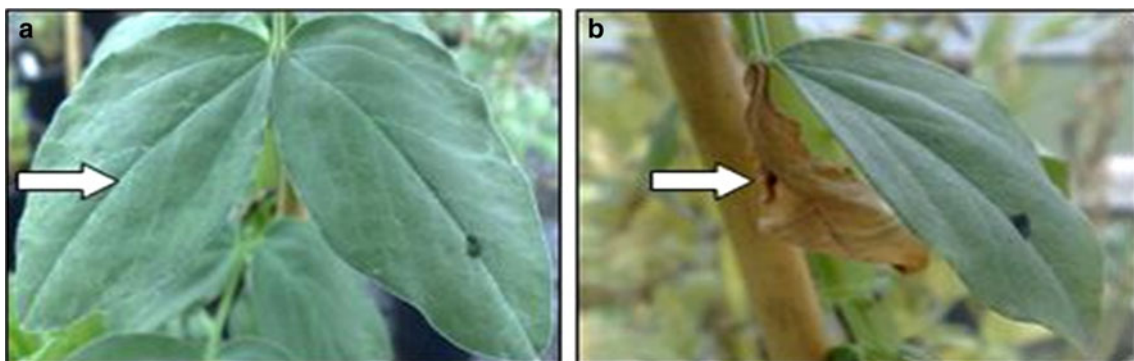
following primers were used ELF1AF 5'-GTGAAGCCCGG-TATGCTTGT-3' and ELF1R 5'-CTTGAGATCCTTGACT GCAACATT-3' (Gutierrez et al. 2011). Gene-specific amplification was evaluated by melting curve analysis and agarose gel electrophoresis. Single melt peaks were obtained for each gene. No primer dimers or other PCR artifacts were detected.

## Results

### Generation of *PR10a*-transgenic faba bean lines

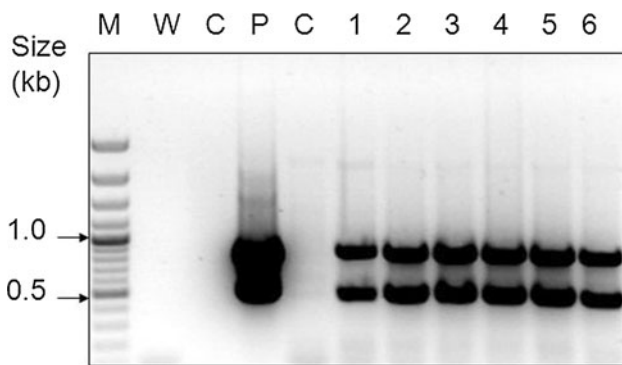
Faba bean embryo axes were transformed with a dicistronic transformation vector containing the potato *PR10a*, which was linked by an IRES sequence to the *Luc* reporter gene. Out of five transformation experiments using 1,500 explants of faba bean cv. *Tattoo*, 10 transgenic shoots representing 4 clones were recovered from all experiments under selection with 2 mg/l PPT. The shoots were selected over a period of 4–6 months and were grafted in vitro and finally transferred to the greenhouse to set seeds. Neither the putative transformants (4 clones) nor subsequent generations of transgenic plants (2 clones, i.e., TL-2 and TL-3) exhibited any phenotypical abnormalities compared with WT (non-transgenic) control plants. Transgenic plants (T0–T3) grown in the greenhouse were analyzed by testing the expression of *bar* gene by applying the leaf paint assay. Within 2 days, necrotic spots appeared on the non-transformed leaves. Ten days after BASTA<sup>®</sup> application, the treated leaflets of transgenic lines showed complete tolerance, in contrast to leaflets of non-transformed plants, which were completely necrotic (Fig. 2).

The stable integration of the T-DNA was confirmed in different clones and in different generations up to T3 by PCR using different primer sets. All clones tested showed the expected band of 480 bp for the *PR10a* gene, while no fragment was amplified in the non-transformed control DNA samples (WT). Inheritance of the transgenes was

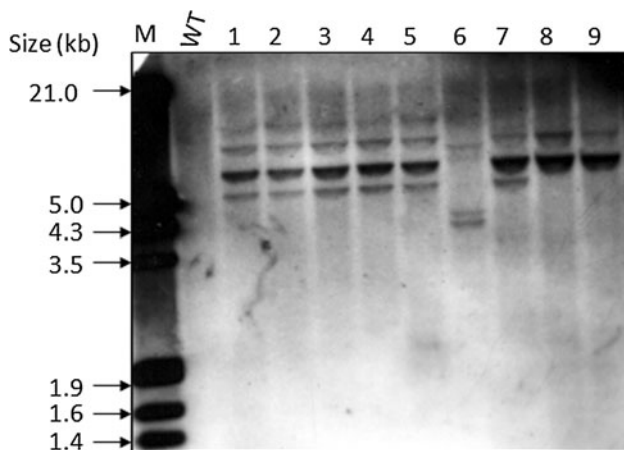


**Fig. 2** Herbicidal leaf paint test showing the resistance of transgenic leaf to BASTA<sup>®</sup> application (300 mg/l ammonium glufosinate) (a), the control non-transgenic leaf dying after BASTA<sup>®</sup> application (b)





**Fig. 3** PCR analysis of *luciferase* and *PR10a* genes in T1 transgenic plants. Lanes *M* 100 bp plus DNA molecular weight marker (MBI Fermentas, St. Leon-Rot, Germany), *W* water, *C* non-transformed control plants, *P* plasmid, lanes 1–3 transgenic plants from line TL-2, lanes 4–6 transgenic plants from line TL-3. The size of the amplified fragment is 834 and 480 bp for *luciferase* and *PR10a* genes, respectively



**Fig. 4** Southern blot analysis of transgenic plants of faba bean. 20  $\mu$ g *Bam*HI-digested genomic DNA, isolated from transgenic faba bean cv. *Tattoo*. Lanes 1–5 representing putative transgenic (T0) plants of line TL-2, lane 6 representing putative transgenic of line TL-3, lanes 7–9 representing stable transgenic plants of T1 of line TL-2 blotted on nylon membrane and probed with *PR10a* probe. *WT* control (non-transformed) plants, *M* Dig-labeled DNA molecular weight marker

confirmed by PCR until T3. Figure 3 shows multiplex PCR results of different samples from different clones that were positive for the *PR10a* and *Luc* genes showing expected fragments of 480 and 834 bp, respectively. All of the PPT-resistant plants exhibited bands of the expected sizes of 447 bp corresponding to *bar* gene (data not shown).

Southern blot analysis was performed to assess the independence of the transgenic plants on the basis of the pattern of transgene integration. Total genomic DNA was digested with *Bam*HI, which cuts the vector only at one site (Fig. 1). The resulting DNA fragments were subjected to hybridization with a probe specific for *PR10a*. Two

independent transgenic lines, TL-2 and TL-3, were thus identified (Fig. 4) and advanced for further investigations.

Segregation for the presence of transgenes was observed in the T1 plants derived from the primary transgenic plants (T0). The two transgenic lines manifested stable integration of the transgenes in the T2 generation and different integration patterns, yielding two to four bands for each transgene. The same banding patterns were observed with the probes for *Luc*, indicating that both transgenes were integrated at the same positions (data not shown).

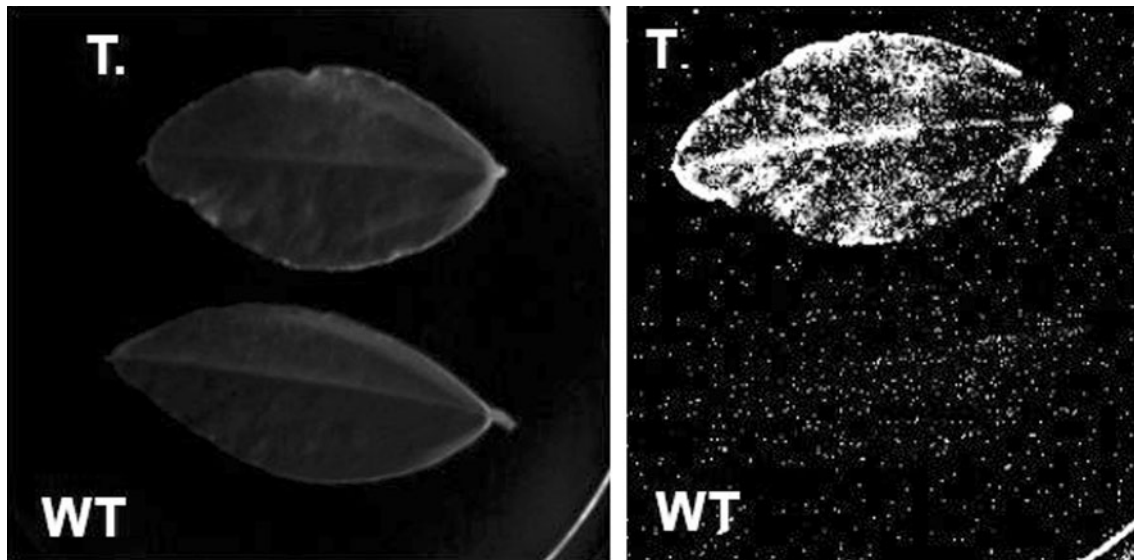
Luciferase activity was observed optically by a high sensitive CCD camera after application of D-Luciferin in all of the PPT-resistant plants with different levels (data not shown). Figure 5 shows the *Luc* expression in the leaves from one of the transgenic faba bean plants (TL-3), in contrast to the non-transformed plants, which did not show any *Luc* expression. In all generations analyzed so far, *Luc* activity was detected with varying luminescence intensities.

RT-PCR analysis indicated that *PR10a* was transcribed in the transgenic lines. The amplification of a 480-bp DNA fragment derived from *PR10a* cDNA is shown for two transgenic lines in Fig. 6a. No signal was obtained with cDNA from non-transgenic plants. The amplification of a 631-bp band from cDNA of *actin1* gene (lacking the 200 bp intron) as shown in Fig. 6b proves the absence of gDNA contamination.

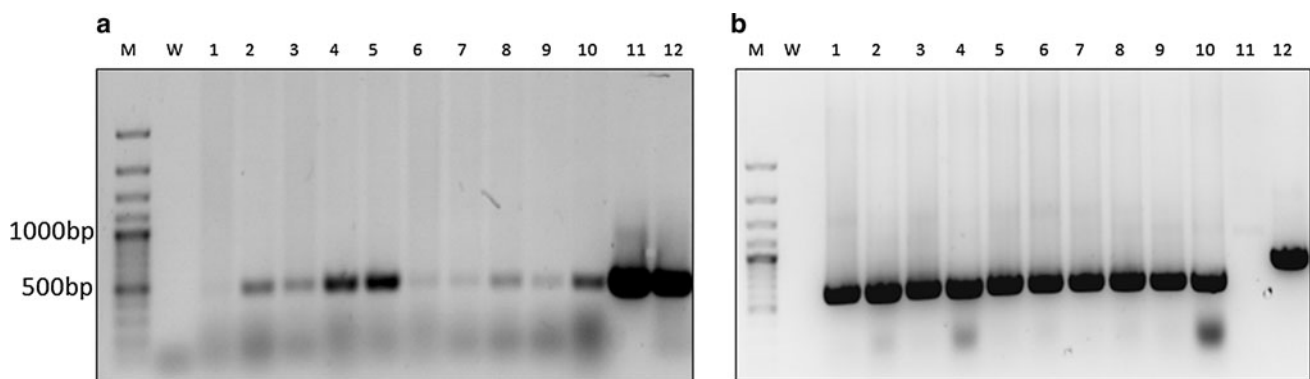
#### Characterization of drought/salt tolerance of transgenic faba bean plants

Four-week-old control and *PR10a*-expressing soil-grown plants were used to validate enhanced stress tolerance of the transgenic plants. Using nine plants under the conditions applied, drought stress by withholding water for 3 weeks caused a severe damage in control plants and most of the plants died. In contrast, most of the TL-3 plants still survived under drought stress although they grew relatively smaller as compared to the non-stressed transgenic controls (Fig. 7a, b). After re-watering, all the surviving TL-3 plants recovered and grew well, but the severely stressed control plants (WT) were unable to recover (Fig. 7c, d).

The plants were then treated with 200 mM NaCl, TL-2, TL-3; control plants exhibited clear growth retardation compared with unstressed plants. Leaves of control plants became yellow and 90 % died after 4 weeks of stress application, but more than 90 % of the leaves of lines TL-2 and TL-3 plants were still green (Fig. 8). For the T2 plants grown in perlite, 9 plants were removed from the soil and the roots were soaked in 150 mM NaCl solution; the control plants showed clear wilting compared with *PR10a*-expressing transgenic plants after 23 h of stress application



**Fig. 5** Luciferase activity in the transgenic (*T*) and wild-type (*WT*) leaves. The *left* image shows the leaf explants under dim light; the *right* image shows the chemiluminescence of the transgenic plants



**Fig. 6** RT-PCR analysis of *PR10a* (a) and *actin1* (b) transcription in the leaves of independent transgenic faba bean plants. *PR10a* transcript resulted in the amplification of 480 bp fragment; *actin1* transcript resulted in the amplifications of 631 bp in all cDNA samples tested (lacking the intron sequence of 200 bp), while the

amplification of gDNA was 831 bp. Lanes *M* 100 bp plus DNA molecular weight marker, *1* non-transformed control plant (*WT*), lanes *2–4* transgenic plants from clone TL-3, lanes *5–10* transgenic plants from clone TL-2, *11* pGIIPR10-cp-luc plasmid as positive control, *12* gDNA from transgenic plant, H<sub>2</sub>O water control

(data not shown). The results indicate that expression of *PR10a* in faba bean plants can improve their tolerance to salt and drought stresses.

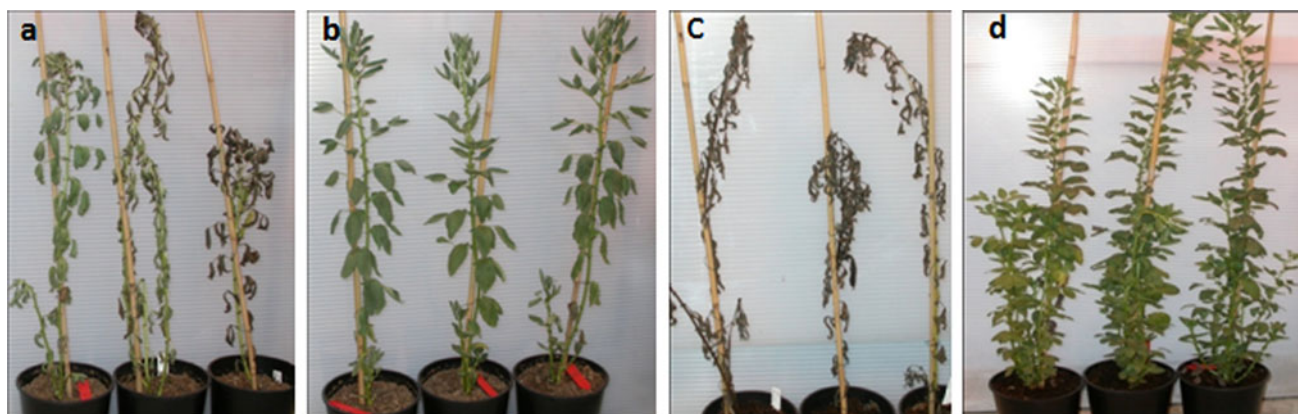
#### Biochemical characterization of transgenic cells under salt stress

The *PR10a*-expressing transgenic plants showed significantly different responses concerning proline accumulation. This was demonstrated by growing three plants of transgenic and wild-type plants under salt stress conditions (200 mM NaCl) with the experiments repeated three times. The proline content in the transgenic plant was about 4.6  $\mu\text{M/g}$  fresh weight compared to 1.6  $\mu\text{M/g}$  in *WT* under

stress condition (Fig. 9), indicating a link between expression of *PR10a* and proline accumulation.

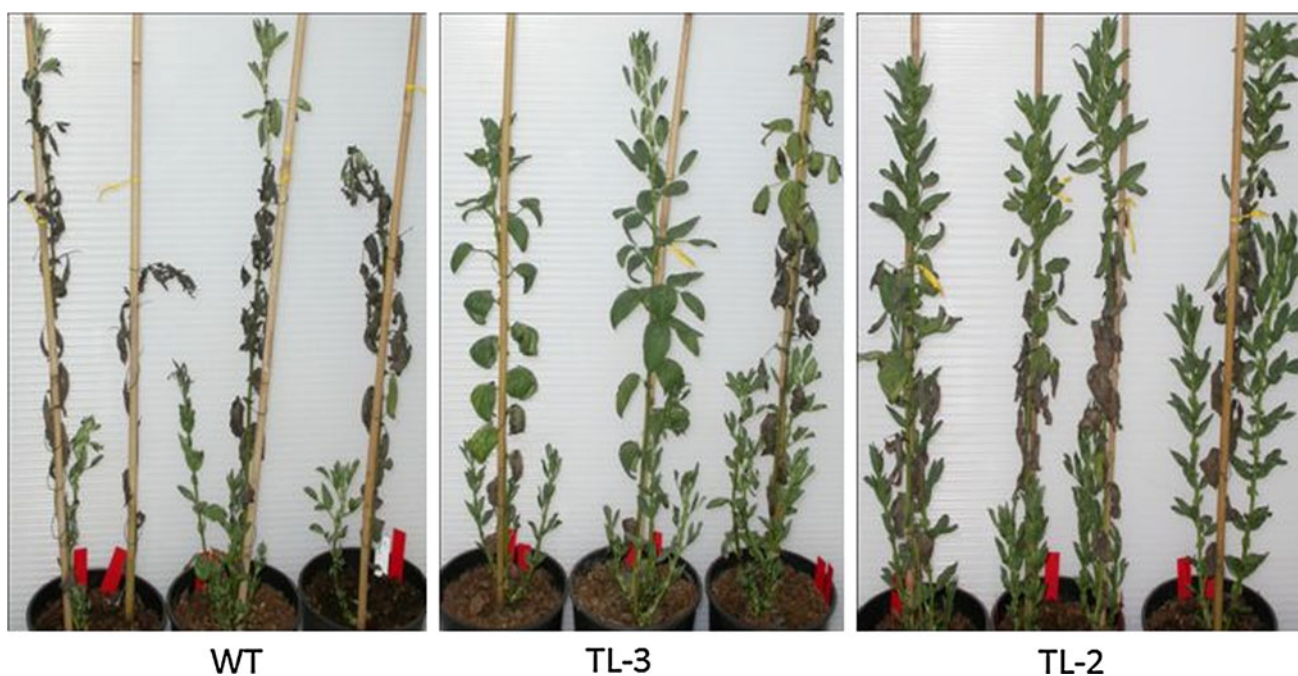
#### Analysis of *PR10a* expression profiles

The transcripts of *PR10a* (Fig. 10a) were detected in the leaves of transgenic lines TL-2 and TL-3. The expression level of *PR10a* gene was higher in transgenic plants under stress than in non-stressed transgenic plants, indicating that the MAS promoter is stress-inducible. No *PR10a* expression was observed in non-transgenic control samples, while comparable *actin1* expression was detected in all cDNA samples (Fig. 10a). Figure 10b presents the normalized fold expression of *PR10a* gene of the transgenic lines under



**Fig. 7** Wild-type (a) and *PR10a* transgenic faba bean plants cv. *Tattoo* (b) deprived of water. Wild-type plants and *PR10a*-expressing transgenic line TL-3 were grown on soil in a 16/8-h day/night photoperiod at 25 °C. Plants were watered every 2 days for 4 weeks.

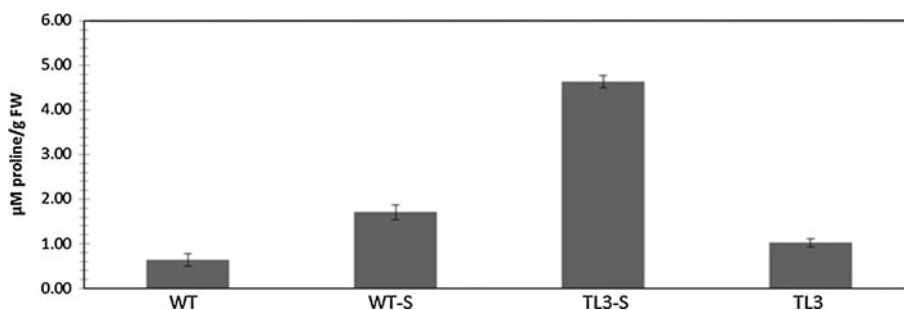
At week 5, the plants were watered once with no further addition of water. The photograph corresponds to plants at 3 weeks of water-deficit stress. Wild-type (c) and *PR10a* transgenic plants line TL-3 (d) after 3 weeks of re-watering



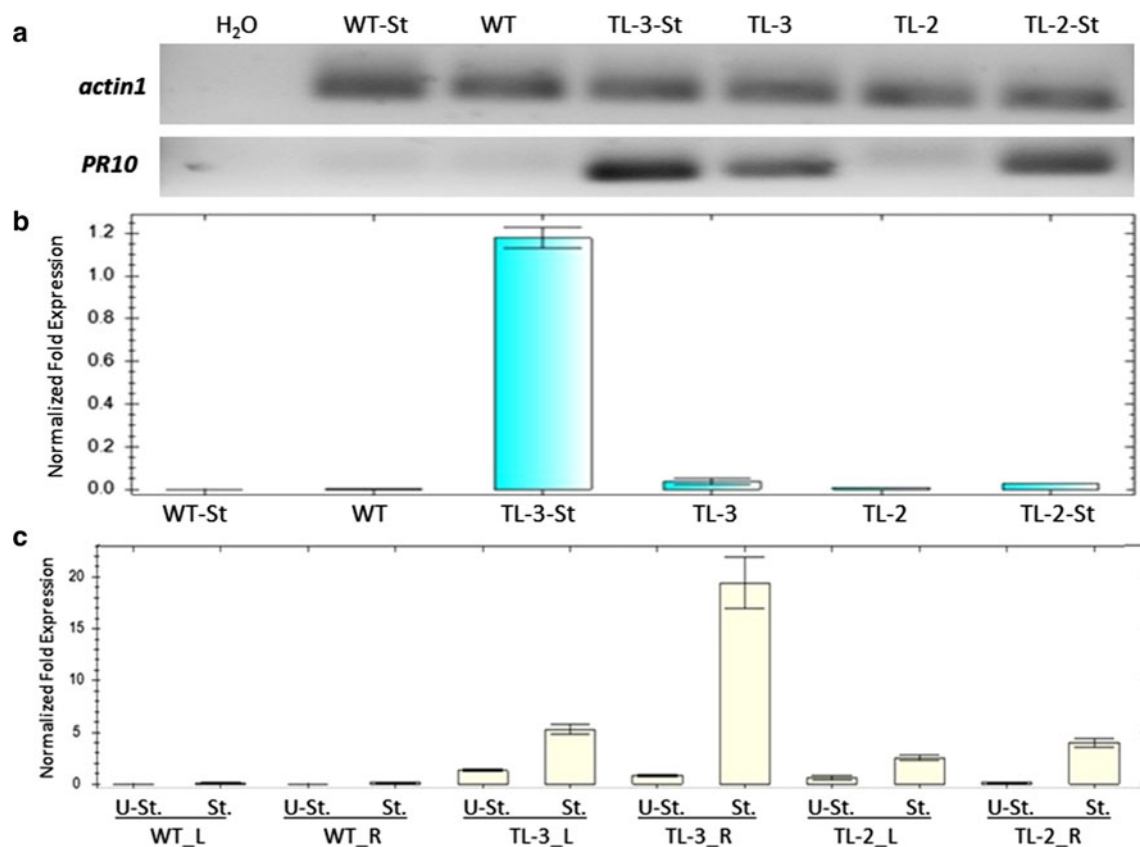
**Fig. 8** Salt treated wild-type (WT) and transgenic faba bean lines (TL-3 and TL-2). Plants were grown on soil in a 16/8-h day/night photoperiod at 25 °C. The plants were watered every 2 days for

4 weeks. At week 5, the plants were treated with 250 ml of 200 mM NaCl every third day. The photograph corresponds to plants at 6 weeks of salt treatment

**Fig. 9** Proline content in the leaves of wild-type and transgenic plants grown under normal condition (WT and TL-3) or at 200 mM NaCl concentration (WT-S and TL3-S), respectively







**Fig. 10** Analysis of *PR10a* gene expression in two transgenic faba bean lines (TL-2 and TL-3) by real-time PCR. **a** Gel electrophoresis of real-time PCR products using *actin1* as a reference gene and *PR10a* specific primers under 200 mM NaCl stress (St lanes) and non-stressed condition, **b** expression levels in unstressed and stressed

(St) faba bean with 200 mM NaCl, **c** expression analysis in leaves (L) and roots (R) in unstressed (USt) and stressed (St) faba bean for 23 h with 150 mM NaCl solution. Expression levels were normalized using *actin1* gene as the reference gene. Normalized fold expression data (mean  $\pm$  SE) represented the mean of three replicates

salt and non-stressed conditions. The expression levels of *PR10a* were found to be significantly (at 5 % level) higher with a maximum of 31-fold in the stressed transgenic plants (TL-3) than that in the non-stressed transgenic plants. No expression was detected in either stressed or non-stressed WT.

The expression levels of the *PR10a* gene in different plant organs (leaves and roots) under stress were also investigated. For this purpose, the expression pattern of *PR10a* under various stress treatments in leaves and roots was investigated (Fig. 10b). Under salt stress, the expression level increased at maximum of 24-fold in roots and 4-fold in the leaves of transgenic plants after 23 h of application and further increased with prolonged stress time course (3 days) with 2,144- and 47-fold increase in the roots and leaves of line TL-3, respectively. In general, the expression levels in the roots under salt stress were found to be much higher than in the leaves (Fig. 10c).

Under air drying treatment, the expression of *PR10a* was also increased after 4 h of treatment in leaves and roots. *PR10a* expression in the leaves of line TL-3 was increased

2- and 62-fold after 4 and 20 h of air drying, respectively. However, the expression in the roots of TL-3 was increased 1.3-fold after 4 h air drying treatment. *PR10a* expression in line TL-2 was increased 13- and 23-fold in the leaves after 4 and 20 h of air drying treatment, respectively. While the expression of *PR10a* in the roots of line TL-2 was increased 45-fold after 4 h of air drying treatment. Due to complete drying of the roots, we could not study the *PR10a* expression in the roots after 20 h of air drying.

## Discussion

### Generation and characterization of transgenic faba bean plants

Although several stable transformants of grain legumes have been described (Popelka et al. 2004; Eapen 2008; Solleti et al. 2008; Ishimoto et al. 2010), the production of transformed faba bean remains difficult. Faba bean is an important grain legume, e.g., in West Asia and North

Africa (WANA) Region. Limited research groups are working on the use of gene transfer for faba bean improvement (Böttinger et al. 2001; Hanafy et al. 2005; Hanafy et al. 2008). We introduced a *PR10a* gene from potato into faba bean in order to improve the drought/salt tolerance of this important grain legume. The *Agrobacterium*-mediated transformation protocol used (Hanafy et al. 2005) has an advantage in reducing the time for recovering transgenic plants to 9 months compared to 16–24 months using *de novo* transformation protocol (Böttinger et al. 2001).

To our knowledge, we report here for the first time the recovering of transgenic faba bean plants expressing a *PR10a* gene from potato. We also demonstrated that the *PR10a* gene is expressed ubiquitously in the transgenic faba bean leaves and roots under non-stressed condition (Fig. 6). Different transgenic faba bean lines of the variety *Tattoo* were recovered and produced seeds. All of the transgenic faba bean lines expressing *PR10a* were fertile and advanced to the T3 generation without any visible detrimental effect on plant growth. Stable integration and inheritance of the introduced genes to subsequent generations were proved by PCR, leaf paint test, *Luc* activity test and Southern blot analysis. Based on the analysis of *PR10a* transgenic lines in this work and the studies published previously (Hashimoto et al. 2004; Srivastava et al. 2004; El-Banna et al. 2010; Vaas et al. 2012; Jain et al. 2012), it is clear that the *PR10a* expression level was enhanced by drought/salt conditions and improved the tolerance to osmotic stress.

Most of PR10 proteins are known to be induced by various biotic and abiotic stresses, and constitutive expression of these proteins was also reported (Awade et al. 1991; Jellouli et al. 2010), suggesting their role in plant defense and development. Many of the proteins of the PR10 family have first been isolated from cell cultures, such as PR10 proteins from parsley (Somssich et al. 1986), peanut (Jain et al. 2006) or soybean (Crowell et al. 1992). PR10a protein was previously detected after osmotic and salt stress as the most prominent *de novo* induced protein spot in the proteome of cell cultures established from potato plants cv. Desiree (El-Banna et al. 2010). The transgenic potato cell line overexpressing *PR10a* gene showed better survival under salt and osmotic challenge than the non-transgenic (El-Banna et al. 2010) as well as during cryopreservation process of potato cell culture (Vaas et al. 2012).

Our results showed that transgenic plants expressing *PR10a* confer enhanced tolerance against salt and drought stresses. Generally, all the transgenic plants tested showed better growth than the WT control plants under induced salt stress of 200 mM NaCl. The leaves were much greener in the transgenics, whereas the leaves of the WT plants were brown and wilted-dry under salt stress (Fig. 8). Previous

studies demonstrated that constitutive expression of a pea PR10 protein enhanced the *Brassica napus* germination under saline conditions (Srivastava et al. 2004). Also, transgenic tobacco plants overexpressing *AhSIPR10* gene from peanut showed higher tolerance to salt, mannitol and heavy metal (Jain et al. 2012). Moreover, through this study, we found that the potato *PR10a* gene enhanced the drought tolerance ability of transgenic faba bean, showing that *PR10a* gene may influence abiotic stress tolerance (Fig. 7). Generally, WT faba bean displayed growth retardation and lethality when treated with high salt concentration or water deprivation, whereas the transgenic plants survived and continued a normal development after stress removal.

In response to environmental stresses, some plants accumulate soluble osmotic molecules, such as proline (Liu and Zhu 1997; Armengaud et al. 2004) as osmoprotectants. Proline is known to be the most widely distributed osmoprotectant, with the highest water solubility among amino acids, and under stress conditions, it functions to adjust osmotic balance, to maintain stability of proteins, membranes and subcellular structures, and to remove reactive oxygen species (ROS) (Zhou et al. 2009). Several studies have demonstrated proline accumulation is positively related to plant tolerance to stresses (Hong et al. 2000). Banu et al. (2009) have reported that proline and betaine protect plants from NaCl-induced cell death by decreasing ROS accumulation. In the present study, it was found that *PR10a* transgenic faba bean plants produced significantly more proline (about 3-fold more than WT control) which presumably protected the transgenic plants from damage as a consequence to the salt stress treatments (Fig. 9). On the other hand, the WT control plants could not tolerate the same degree of stress. These results are in accordance with those of El-Banna et al. (2010), Agarwal and Pandey (2004) and Dash and Panda (2001).

Differential expression of potato *PR10a* in response to abiotic stress

We noticed that line TL-3 exhibited higher expression levels of *PR10a* than line TL-2 in the leaves under non-stressed conditions. MAS promoter was considered to be constitutive promoter, but it was mentioned that it is also induced by wounding and auxin/cytokinin treatment (Langridge et al. 1989; Guevara-García et al. 1993). Our study showed that *PR10a* under the MAS promoter is enhanced by drought and salt stresses in the leaves and roots of the transgenic plants. However, different transgenic events may have differences in the expression level and stress tolerance levels as seen between lines TL-3 (highest expression level of *PR10a* with higher stress tolerance) compared to line TL-2 (low expression level of

*PR10a*). Our findings are in accordance with the previous report of EL-Banna et al. (2010) using the same promoter. They reported that overexpressing *PR10a* in transgenic potato tissues led to enhanced salt and osmotic tolerance up to 500 mM sorbitol and 200 mM NaCl without growth reduction.

In the present study, when transgenic faba bean plants were subjected to salt stress, the expression level of *PR10a* in the roots was much higher than the expression levels in the leaves (Fig. 10c). In contrast, when the transgenic plants were subjected to air drying, the expression level of *PR10a* in the leaves was significantly higher than the levels detected in the roots. Similarly, Przymusinski et al. (2004) observed increased levels of the *RzPR10* protein in lupine roots exposed to 500 mM NaCl for 48 h. Comparable results on increased levels of PR10 protein were reported in peanut callus cells obtained from leaves and subjected to a 15-day salt stress (Jain et al. 2006), while significant changes in abundance of PR10 in pea roots occurred after 6 weeks of treatment with 150 mM NaCl (Kav et al. 2004).

Our observation that potato PR10a expression is induced upon salinity and drought stress supporting the hypothesis that this protein may have a significant role in protecting plant cellular components from the deleterious effects of salinity and drought (Kav et al. 2004). Our findings suggest that transformation with this gene can be a feasible strategy to improve drought/salt stress tolerance of legume plants. However, the precise roles of the PR10a protein in abiotic stress responses are not fully clear. Further investigations are needed to clarify the molecular mechanisms of stress tolerance involving the PR10a protein.

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