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ORIGINAL ARTICLE

Evaluation of abiotic stress tolerance and physiological characteristics of potato (*Solanum tuberosum* L. cv. Kennebec) that heterologously expresses the rice *Osmyb4* gene

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Abstract MYB transcription factors are involved in diverse biochemical and physiological processes, including hormone signaling, defense, and stress responses. In the present study, we developed a transgenic potato (*Solanum tuberosum* L. cv. Kennebec) expressing the rice *Osmyb4* gene, which encodes the transcription factor MYB4. The transgene was under the control of either the constitutive *CaMV35S* promoter or the stress-induced *Arabidopsis COR15a* promoter. The potential involvement of MYB4 in certain physiological processes and the abiotic stress response in the potato was evaluated. The transgenic plants did not exhibit growth retardation, and they showed no significant difference ($P < 0.05$) in tuber yield from that of non-transgenic wild-type plants. Although the chlorophyll *a* and *b* as well as the anthocyanin contents of the six transgenic lines were similar to those of the wild type, the transgenic line S2 presented a significantly higher carotenoid content. The total sugar contents of the lines S2 and M48 were significantly higher than that of the wild-type plants. S2 and M48 were significantly more tolerant of salinity than the wild type, according to measured growth parameters. Transgenic plants grown under a high concentration of boric acid (3 mM) exhibited greater survival rates than non-transgenic control plants. On the other hand, the transgenic plants did not show an improvement in freezing tolerance. Overall, our results indicated that MYB4 may affect diverse processes such as carotenoid biosynthesis, sugar metabolism, and salinity tolerance in potato, and that it may be an upstream regulatory element of these processes.

Keywords Abiotic stress · MYB4 · Transcription factor · Transgenic potato

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Introduction

The MYB family of transcription factors (TFs) is one of the most populous classes of transcription factors in plants. MYB proteins can be classified into four subfamilies (MYB-1R, R2R3-MYB, MYB-3R, and MYB-4R) depending on the number of adjacent repeats in the MYB domain. R2R3-MYB is the largest and functionally the most diverse subfamily, with more than 100 members. MYB TFs play important roles in the regulation of anthocyanin biosynthesis, the determination of cell shape, and the formation of different plant organs. They are also involved in responses to biotic and abiotic stresses and certain hormone signals (Chen et al. 2005; Dubos et al. 2010). A total of 8,746 MYB TFs have been identified in plants, most of which have been described in *Glycine max* (369 TFs), *Musa acuminata* (303 TFs), *Gossypium raimondii* (299 TFs), *Populus trichocarpa* (266 TFs), *Brassica rapa* (249 TFs), and *Malus domestica* (238 TFs). To date, 126 MYB TFs and 107 MYB-related TFs have been

identified in *Solanum tuberosum* (Jin et al. 2014). However, studies demonstrating the function of MYB TFs in potato are relatively rare. Baranowskij et al. (1994) isolated and characterized a potato cDNA encoding a novel sequence-specific DNA-binding protein, designated MybSt1. The gene contained a small region with homology to the DNA-binding domain of the proto-oncogene c-Myb. Transient expression systems demonstrated that MybSt1, the first reported MYB-1R TF in plants, functions as a transcriptional activator. Shin et al. (2011) demonstrated the localization of StMYB1R-1 to the nucleus and confirmed its function as a transcription factor. Overexpression of *StMYB1R-1* in transgenic potato enhanced the expression of drought-regulated genes such as *AtHB-7*, *RD28*, *ALDH22a1*, and *ERD1*-like, and improved plant tolerance to drought stress. Rommens et al. (2008) demonstrated activation of the phenylpropanoid biosynthetic pathway via tuber-specific expression of the native and slightly modified MYB transcription factor gene $StMtf1^{M}$ in potato. Caffeoylquinates, various flavonols, and anthocyanins were accumulated in higher amounts in transgenic tubers compared to untransformed controls.

The R2R3-type gene *Osmyb4* exhibits a high sequence similarity to MYB TFs identified in *Triticum aestivum* (64 %), *Hordeum vulgare* (29 %), *Leymus racemosus* (29 %), *Leymus multicaulis* (29 %), and *Zea mays* (28 %). Overexpression of *Osmyb4* confers cold and drought tolerance in *Arabidopsis*, apple, sage, and tobacco through the accumulation of several compatible solutes and the induction of phenylpropanoid biosynthesis (Vannini et al. 2004; Mattana et al. 2005; Docimo et al. 2008; Pasquali et al. 2008). Although the *Osmyb4* gene was shown to activate stress-responsive pathways in different species, it was reported that its effect on stress tolerance may differ according to the genetic background of the transformed species (Vannini et al. 2007; Pasquali et al. 2008).

In the study reported in the present paper, transgenic potato plants expressing the *Osmyb4* gene under the control of the constitutive *CaMV35S* promoter or the cold-inducible *COR15a* promoter were generated to elucidate the metabolic pathways that might be regulated by MYB4 TF in potato. The growth, tuber yields, pigment contents, and sugar contents of the transgenic plants were evaluated with respect to those of the wild type (WT) to assess the effect of heterologous *Osmyb4* expression on growth and physiological characteristics. The growth parameters of WT and transgenic plants subjected to 100 mM NaCl, 3 mM boric acid, and freezing temperatures were compared to evaluate the potential involvement of MYB4 in the regulation of abiotic stress tolerance in potato.

Materials and methods

Plant materials and growth conditions

Solanum tuberosum cv. Kennebec was used for transformation. Leaves of plantlets, regenerated in vitro from single node cuttings on MS (Murashige and Skoog 1962) basal medium supplemented with 30 g/L sucrose for 1 month, were used in transformation experiments. Transgenic plantlets were propagated on MS basal medium supplemented with sucrose. The cultures were maintained at 23 ± 2 °C with a 16-h light (400 µmol m^{-2} s^{-1}) and 8-h dark photocycle.

Agrobacterium strain, vectors, and potato transformation

Transformation was carried out using *Agrobacterium tumefaciens* strain EHA105 harboring two different binary vectors containing the *Osmyb4* gene (accession number Y11414)—which encodes the MYB4 transcription factor—under the control of either the *CaMV35S* promoter (pCaMVmyb4) or the cold-inducible *COR15a* promoter (pCOR15amyb4). The vectors contained *nptII* as the selectable marker. The plasmids were kindly provided by Immacolata Coraggio (Istituto di Biologia e Biotecnologia Agraria, Milano, Italy). Potato leaves removed from 3- to 4-week-old shoots were cut into 2–3 mm wide strips and transformed according to a modified procedure of Wenzler et al. (1989). First of all, the excised leaf explants were placed abaxial side down on Petri plates containing media rich in hormones (MS + 30 g/L sucrose + 10 mg/L NAA + 10 mg/L ZR + 3 g/L phytagel) and incubated for 3 days in the dark. Following this pre-treatment, the leaf strips were co-cultivated with a suspension (diluted 1:30 with liquid MS) of a saturated liquid culture of *Agrobacterium* for 15 min and transferred to Petri plates containing LSR-1 media free of kanamycin (MS + 30 g/L sucrose + 0.2 mg/L NAA + 2 mg/L ZR + 0.02 mg/L GA_3 + 3 g/L phytagel). After a second incubation for 3 days in the dark, the explants were transferred to LSR-1 plates containing 100 mg/L kanamycin and 300 mg/L timentin. After callus formation, the explants were transferred to LSR-2 media (MS + 30 g/L sucrose + 2 mg/L ZR + 0.02 mg/L GA_3 + 3 g/L phytagel) supplemented with antibiotics for shoot formation. Plantlets regenerated on selective LSR-2 media were referred to as putative transgenic lines. These putative lines were propagated on MS basal medium for further use. Transgenic plants expressing *Osmyb4* under the control of the *COR15a* promoter were designated "pCOR lines," and transgenic plants expressing *Osmyb4* under the control of the *CaMV35S* promoter were designated "pCaMV lines." The efficiency of transformation

was calculated as the number of shoots which developed roots on selective media divided by the total number of explants used for transformation.

RNA isolation and northern blot analysis

Total RNA was isolated from leaf tissues using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. pCOR lines were incubated at 4 °C for 3 days prior to RNA isolation in order to induce the *COR15a* promoter. Northern blot analysis of the wild-type and randomly selected putative transgenic lines was performed according to the standard protocol (Sambrook et al. 1989). Ten micrograms of total RNA were loaded onto a 1 % agarose–formaldehyde gel and blotted onto a Hybond-N membrane (Amersham Biosciences, Piscataway, NJ, USA). Hybridizations were performed at 42 °C using a DIG-labeled *Osmyb4* probe prepared using the PCR DIG Labeling Mix (Roche Applied Science, Basel, Switzerland). The PCR amplification was carried out using *Osmyb4*-specific primers (F:CGAGAAGATGGGGCTCAAG and R:TCGGCTTCTTGTGCTTCTTGC). The *Osmyb4* gene-specific probe was a 389-bp fragment spanning the 5′ coding region. Hybridization signals were detected with the CDP-Star reagent (New England Biolabs, Beverly, MA, USA).

Determination of tuber yield

Four-week-old in vitro grown transgenic and WT plants were planted in pots containing peat and soil (1:1) and transferred to a greenhouse. After 7 weeks of growth at 25 °C, the transgenic lines and the WT were phenotypically evaluated for growth performance. The tubers were harvested after 4 months. Numbers and weights of tubers were determined to evaluate the tuber formation potencies of the WT and transgenic lines.

Determination of pigment and sugar contents

Leaf discs collected from fully expanded third and fourth leaves of the WT and transgenic lines grown in the greenhouse for 1 month were used to determine chlorophyll (Chl), total carotenoid, and anthocyanin contents. Leaf discs were agitated gently in the dark for 24 h at 4 °C in 1 mL 80 % (v/v) acetone and the extracts were centrifuged. The absorbance of the supernatant was measured at 470, 657, and 663 nm using a spectrophotometer. Chlorophyll *a*, chlorophyll *b*, total chlorophyll, and total carotenoid concentrations were calculated according to the equations given by Lichtenthaler (1988). Anthocyanins were extracted in 3 M HCl:H_2O:MeOH (1:3:16, v/v) and concentrations were estimated as $A_{530} - 0.24\,A_{653}$. The anthocyanins absorbed maximally at 530 nm and the subtraction of 0.24 A_{653} compensated for the small overlap in absorbance at 530 nm with the chlorophylls (Murray and Hackett 1991).

The total sugar contents in the WT and transgenic lines were determined according to Amirjani (2011). Leaf samples collected from plants grown in the greenhouse for 1 month were oven dried at 80 °C for 48 h and homogenized in 80 % (v/v) ethanol. The homogenates were placed in a water bath at 80 °C for 30 min and then centrifuged at $3000\times g$ for 5 min. The samples were washed twice with H_2O at room temperature and resuspended with 3 mL H_2O and boiled for 2 h. Contents of total sugars were estimated colorimetrically using the phenol sulfuric acid method described by Dubois et al. (1956).

In vitro application of salinity stress and boron toxicity

Single node cuttings from transgenic and WT plantlets grown on MS basal medium until the 5–6 leaves stage were used for salinity stress and boron toxicity treatments. Thirty-two single node cuttings from each transgenic line and the WT were cultured on MS basal medium supplemented with 100 mM NaCl or 3 mM boric acid for 30 days. pCOR lines were incubated at 4 °C for 3 days prior to stress treatment to induce the *COR15a* promoter. Survival rate, shoot length, root length, and shoot dry weight were determined to evaluate the salinity and boron toxicity tolerances of the transgenic plants. Survival rates were determined according to the number of rooting plantlets on salt or boric acid containing media.

Freezing stress application

Plants grown in the greenhouse for 1 month were cold acclimated at 4 °C for 1 week and subjected to freezing temperatures. The freezing tolerances of the WT and transgenic plants were determined as described by Pino et al. (2007). For each temperature point evaluated, three independent plants and three leaf discs from each plant were used. The leaf discs were collected from fully expanded second and third leaves and placed in test tubes. The samples were incubated at −1 °C for 1 h in a water bath placed in a cooled incubator. Ice nucleation was initiated by adding an ice chip to each tube. The samples were then maintained at −1.5 °C for an additional 1 h, and the temperature was lowered 1 °C/h. Samples were removed at −3, −4, and −5 °C, and slow-thawed overnight at 2 °C. Freezing injuries of the thawed leaf samples were assessed by recording electrolyte leakages using a conductivity meter. Following conductivity measurements, all samples were frozen at −20 °C for 24 h, thawed at room temperature, and used again to determine the total conductivity.

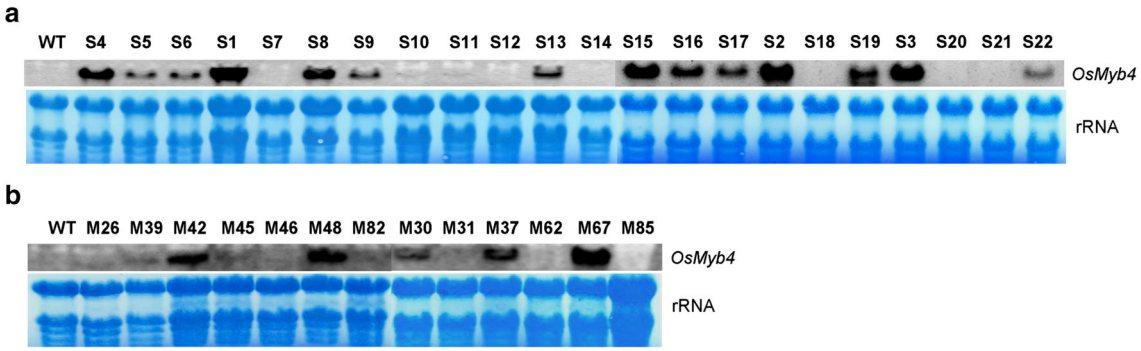

Fig. 1a–b Northern blot analysis of non-transgenic wild-type (WT) and transgenic lines. **a** Expression of the *Osmyb4* gene in pCaMV lines. **b** Expression of the *Osmyb4* gene in pCOR lines. The pCOR lines were incubated at 4 °C for 3 days prior to RNA isolation in order to induce the *COR15a* promoter. Ten micrograms of total RNA extracted from leaves were loaded per lane. Blot Stain Blue (Sigma)-stained rRNA is shown below the RNA blots (*rRNA*)

Statistical analysis of data

The SPSS software program was used to determine the differences between the WT and transgenic lines. To confirm the variability of the data and validity of the results, all of the data were subjected to an analysis of variance (ANOVA). Least significant differences (LSD) were calculated at the 5 % level ($P < 0.05$) to determine the differences between the mean values for the WT and those for the transgenics. The standard errors of the means were calculated by performing the descriptive statistics test in the same program.

Results and discussion

Generation of transgenic potato lines expressing *Osmyb4*

A total of 201 shoots out of 534 explants transformed with pCOR15a*myb4* developed roots on MS media containing kanamycin. The number of explants that were transformed with pCaMV*myb4* was 80, and a total of 22 shoots developed roots on selective media. The transformation rate of potato with the *Osmyb4* gene was found to be either 37.6 % (pCOR15a*myb4*) or 27.5 % (pCaMV*myb4*), demonstrating the efficiency of the transformation method.

Analysis of gene expression in transgenic plants

The expression of the *Osmyb4* gene was analyzed in 13 randomly selected pCOR lines and 22 randomly selected pCaMV lines, and then compared with WT control plants (Fig. 1). The northern blots hybridized with the *Osmyb4*-specific probe showed various expression levels in transgenic lines. No hybridization signal was observed in the WT. We decided to use four pCOR lines (M37, M42, M48, and M67) and three pCaMV lines (S1, S2, and S3) with the highest levels of expression for further analysis. A low level of *Osmyb4* expression was detected in the pCOR lines even before promoter induction, demonstrating that the promoter is leaky (data not shown).

Effect of ectopic *Osmyb4* expression on plant phenotype and tuber yield

The phenotypes of the WT and transgenic potato lines grown in the greenhouse for 7 weeks were compared to evaluate the effect of *Osmyb4* expression on growth. Overexpression of some transcription factors was reported to lead to growth retardation in transgenic plants (Liu et al. 1998; Kasuga et al. 1999). Although Vannini et al. (2004) reported a dwarf phenotype in transgenic *Arabidopsis* plants overexpressing *Osmyb4*, no growth retardation was observed in transgenic potato lines expressing *Osmyb4* (Fig. 2). Vannini et al. (2004) suggested that the severity of the dwarf phenotype depended on the level of expression. However, not only the pCOR lines but also the pCaMV lines were phenotypically uniform and indistinguishable from the WT potato plants. This indicates that the effect of *Osmyb4* expression on phenotype is independent of the expression level in transgenic potato. The variable responses of *Arabidopsis* and potato suggest that the downstream genes regulated by MYB4 TF may differ depending on the species or the genomic background of the species.

Since potato is grown for its tubers, the impact of ectopic *Osmyb4* expression on tuber formation in transgenic lines was investigated (Table 1). There was no significant difference in the yield and number of tubers in the transgenic lines as compared to the non-transgenic WT. The mean tuber size was higher than that in the WT in four of the seven tested transgenic lines. Pino et al. (2007) reported abolished or reduced tuber yields in transgenic

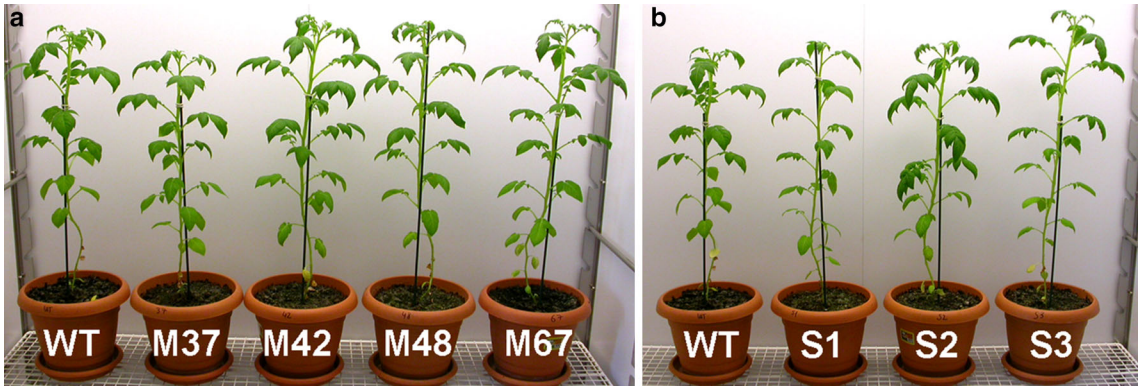

Fig. 2a–b Phenotypes of non-transgenic wild-type (WT) and transgenic lines grown in the greenhouse for 7 weeks. **a** pCOR lines (M37, M42, M48, M67). **b** pCaMV lines (S1, S2, S3). No growth retardation was observed in the transgenic plants as compared to the WT

Table 1 Effect of heterologous *Osmyb4* gene expression on tuber formation

	Number of tubers (no./plant)	Tuber yield (g/plant)	Mean tuber size (g)
Non-transgenic			
WT	2.5 ± 1.38	30.75 ± 11.87	12.30
pCaMV lines			
S1	3.7 ± 1.51	35.06 ± 6.27	9.56
S2	2.2 ± 0.75	34.35 ± 12.7	15.86
S3	3.2 ± 1.47	34.89 ± 17.26	11.02
pCOR lines			
M37	2.4 ± 0.55	24.48 ± 7.51	10.20
M42	2.3 ± 1.51	29.17 ± 12.53	12.50
M48	2.3 ± 1.21	36.10 ± 16.23	15.47
M67	2.3 ± 0.82	32.12 ± 16.37	13.76

Tubers were harvested from 16-week-old plants

WT wild type; data represent mean ± SEM; $n = 6$

Table 2 Survival rates and physiological parameters of non-transgenic wild-type (WT) and transgenic plants grown on MS medium containing 100 mM NaCl

	Survival rate (%)	Shoot length (cm)	Root length (cm)	Shoot DW (mg)
Non-transgenic				
WT	44	0.92 ± 0.13	12.84 ± 1.17	19.12 ± 2.80
pCaMV lines				
S1	50	0.77 ± 0.08	13.49 ± 0.71	7.86 ± 0.58[a]
S2	41	1.98 ± 0.24[a]	12.76 ± 0.98	27.65 ± 2.33[a]
S3	25	1.05 ± 0.16	12.36 ± 1.12	20.58 ± 2.53
pCOR lines				
M37	23	1.59 ± 0.49	10.84 ± 1.65	25.94 ± 3.61
M42	9	0.70 ± 0.06	12.40 ± 5.31	15.90 ± 3.39
M48	45	2.29 ± 0.38[a]	12.00 ± 0.96	27.50 ± 2.29[a]
M67	39	1.40 ± 0.23	13.12 ± 1.14	20.29 ± 1.73

A total of 32 explants were subjected to salinity stress, and only surviving plantlets with roots were evaluated for physiological parameters

DW dry weight; data represent mean ± SEM

[a] Values are significantly different from those of the WT according to an LSD test at $P < 0.05$

potatoes overexpressing *CBF2* and *CBF3* TF genes, which corresponded with a reduction in vegetative growth. In the present study, the heterologous expression of *Osmyb4*, which encodes the MYB4 TF, did not alter the tuber yield, suggesting that neither induced nor constitutive expression of *Osmyb4* had a negative effect on potato tuberization. This may be a result of similar vegetative growth potentials in the WT and transgenic lines.

Effect of ectopic *Osmyb4* expression on salinity and boron toxicity tolerance

The survival rates and physiological parameters of the WT and transgenic lines grown on MS media containing 100 mM NaCl and 3 mM boric acid are given in Tables 2 and 3, respectively. Salinity stress caused a significant reduction in plant growth in both the WT and transgenic lines (Fig. 3). The survival rate of the WT was higher than those of the transgenic lines S2, S3, M37, M42, and M67 (Table 2). However, no significant improvement in the growth of the roots and shoots of the WT was recorded as compared to those of these transgenic lines. The shoot lengths and shoot dry weights of two transgenic lines (S2 and M48) were significantly greater ($P < 0.05$) than those of the WT. There was no significant difference between them in root length (Table 2). The simplest way to analyze the response of a plant to salinity stress is to observe the reduction in shoot growth, which occurs in two phases. The first is the osmotic phase, which starts directly when the salt concentration increases to high levels in the growth environment. In this phase, the rate of shoot growth falls significantly. In the second, ion-specific phase, the salt

concentration reaches toxic levels and consequently the plant dies. Therefore, the salinity tolerance of a plant may be assessed by monitoring its survival and shoot growth rate upon exposure to high concentrations of salt. Tolerant plants grow at a reasonably rapid rate during stress compared to sensitive plants (Borsani et al. 2003; Munns and Tester 2008). The significantly greater shoot lengths and shoot dry weights of transgenic lines (S2 and M48) upon exposure to 100 mM NaCl indicate more rapid growth compared to that of the WT. Vannini et al. (2006) reported increased salinity tolerance in transgenic *Arabidopsis* overexpressing *Osmyb4*, which is in accordance with our results. Following 300 mM NaCl treatment, about 50 % of the transgenic *Arabidopsis* plants survived, but none of the WT plants did.

Table 3 Survival rates and physiological parameters of non-transgenic wild-type (WT) and transgenic plants grown on MS medium containing 3 mM boric acid

	Survival rate (%)	Shoot length (cm)	Root length (cm)	Shoot DW (mg)
Non-transgenic				
WT	9	1.07 ± 0.38	7.10 ± 0.06	7.43 ± 2.94
pCaMV lines				
S1	41	1.24 ± 0.16	1.13 ± 0.21[a]	5.43 ± 0.65
S2	56	1.59 ± 0.27	5.03 ± 0.57	9.67 ± 1.08
S3	34	1.41 ± 0.19	4.72 ± 0.53[a]	5.92 ± 0.55
pCOR lines				
M37	59	1.27 ± 0.16	5.63 ± 0.35	7.83 ± 0.88
M42	19	2.03 ± 0.70	4.32 ± 1.14[a]	9.47 ± 3.87
M48	38	1.50 ± 0.32	3.37 ± 0.34[a]	11.63 ± 2.21
M67	16	1.00 ± 0.21	1.26 ± 0.18[a]	8.48 ± 1.39

A total of 32 explants were subjected to 3 mM boric acid, and only surviving plantlets with roots were evaluated for physiological parameters

DW dry weight; data represent mean ± SEM

[a] Values are significantly different from those of the WT according to an LSD test at $P < 0.05$

Previously, MYB-type TFs were shown to be upregulated in barley under boric acid toxicity (Hassan 2007; Oz et al. 2009). Nozawa et al. (2006) also demonstrated that the expression of two MYB TFs from *Arabidopsis* conferred boric acid tolerance in yeast. In the present study, a possible involvement of MYB4 in the boric acid tolerance mechanism was investigated. Although boron (B) is an essential micronutrient for plant growth, it becomes toxic when present at high levels. B toxicity leads to reduced root cell division (Liu and Yang 2000) and decreased shoot and root growth (Lovatt and Bates 1984; Nable et al. 1990). Physiological growth parameters (such as the lengths and weights of shoots and roots) of plants grown under high B concentrations may indicate B toxicity tolerance. High concentrations of boric acid (3 mM) severely limited growth in both WT and transgenic potato lines (Fig. 4). Although four transgenic lines (S2, M37, M42, M48) showed better growth under boric acid toxicity (Fig. 4), there was no significant difference in shoot lengths and shoot dry weights as compared to WT (Table 3). The root length of WT was significantly higher than those of five out of the seven tested transgenic lines ($P < 0.05$). However, the root growth did not correspond with the shoot growth, as the shoot length and shoot DW of WT were not significantly higher than those of any of the transgenic lines. Only 9 % of WT plantlets survived on the media

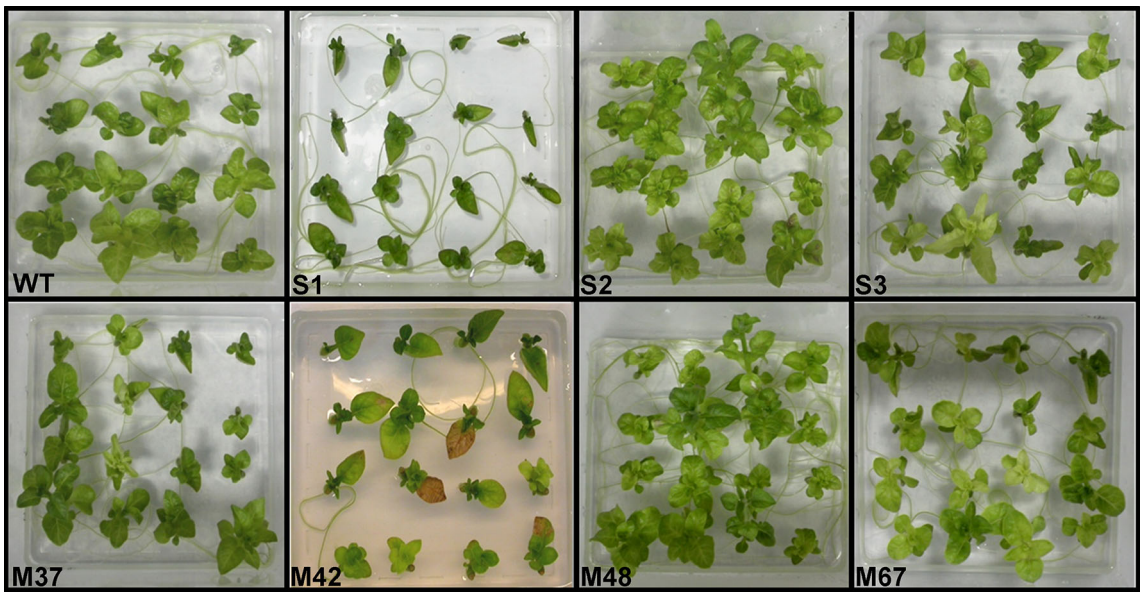

Fig. 3 Effect of 100 mM NaCl on the growth of the wild type (WT), pCaMV lines (S1, S2, S3), and pCOR lines (M37, M42, M48, M67)

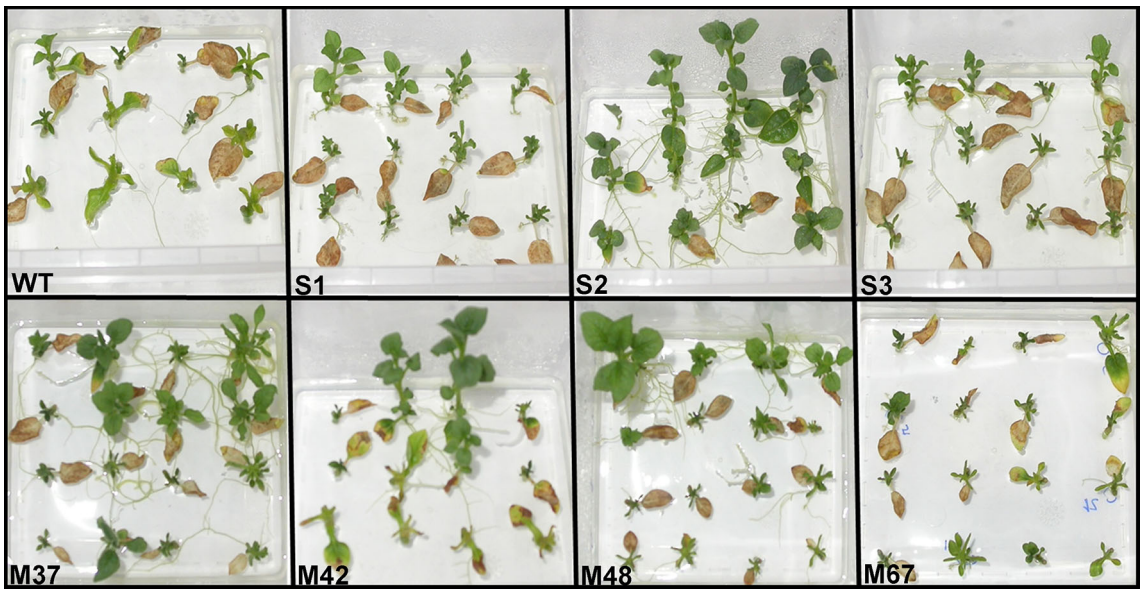

Fig. 4 Effect of 3 mM boric acid on the growth of the wild-type (WT), pCaMV lines (S1, S2, S3), and pCOR lines (M37, M42, M48, M67)

containing boric acid, whereas this rate was much higher in all transgenic lines (Table 3). The large difference in survival rate between the transgenic lines and the WT suggests that heterologous expression of *Osmyb4* might be involved in the B toxicity tolerance mechanism in potato. To the best of our knowledge, the present work provides the first data on the effect of B toxicity on transgenic plants that express *Osmyb4*.

Effect of ectopic *Osmyb4* expression on freezing stress tolerance

Changes in membrane integrity and fluidity are some of the more immediate responses of plants upon exposure to freezing, showing that membranes are sites of perception and/or injury (Sung et al. 2003). Electrolyte leakage is widely used as an indicator of membrane damage generated by various stresses in all structures (Parvanova et al. 2004). In the present study, electrolyte leakages in the WT and transgenic lines were measured to compare the damage caused by freezing temperatures. Table 4 shows the electrolyte leakages of the transgenic lines and the WT. Continuously reducing the temperature led to a gradual increase in membrane damage in terms of electrolyte leakage in both the WT and transgenic lines (data not shown). There were no significant differences in electrolyte leakage between the transgenic lines S1, M37, and M42 and the WT. The electrolyte leakages of S3, M48, and M67 were significantly higher at −3 °C. The transgenic line S2 had significantly greater electrolyte leakage at −4 °C. However, none of the electrolyte leakages of the transgenic lines, except for M67, were significantly different at −5 °C, which was the lowest temperature applied. *Solanum tuberosum* is a frost-sensitive species that is incapable of cold acclimation. It has a maximum freezing tolerance of about −3 °C both before and after exposure to low temperatures (Chen and Li 1980). In the present study, the electrolyte leakage measurements at −3 °C and lower temperatures demonstrated that transformation of potato with *Osmyb4* did not improve freezing tolerance in the transgenic lines.

Contrary to the results obtained in the present study, an increased freezing tolerance was reported for transgenic

Table 4 Electrolyte leakages (%) of the wild-type (WT) and transgenic lines subjected to freezing temperatures

	Electrolyte leakage (%)		
	−3 °C	−4 °C	−5 °C
Non-transgenic			
WT	100	100	100
pCaMV lines			
S1	55.7	74.8	94.4
S2	126.6	153.3[a]	111.4
S3	133.4[a]	101.0	89.6
pCOR lines			
M37	144.1	92.9	84.4
M42	113.6	108.1	103.4
M48	129.5[a]	94.3	104.1
M67	148.4[a]	90.0	78.3[a]

Electrolyte leakages of the transgenic lines are the percentage changes with respect to WT; $n = 9$

[a] Values are significantly different from that of the WT according to an LSD test at $P < 0.05$

Table 5 Pigment and total sugar concentrations in non-transgenic wild-type (WT) and transgenic lines S2 and M48 (S2: pCaMV line; M48: pCOR line)

	Chlorophyll (μg cm^{-2})			Carotenoid (μg cm^{-2})	Anthocyanin (μg cm^{-2})	Sugar (mg g^{-1} DW)
	Chl *a*	Chl *b*	Chl *a* + *b*			
WT	18.21 ± 0.89	6.66 ± 0.26	24.87 ± 1.15	3.77 ± 0.18	0.068 ± 0.013	18.55 ± 1.56
S2	20.65 ± 0.57	7.30 ± 0.18	27.95 ± 0.72	4.45 ± 0.14[a]	0.053 ± 0.009	23.69 ± 1.21[a]
M48	17.56 ± 0.70	6.28 ± 0.22	23.84 ± 0.90	3.78 ± 0.10	0.052 ± 0.005	25.38 ± 0.62[a]

Data represent mean ± SEM; $n = 6$–9

[a] Values are significantly different from that of WT according to an LSD test at $P < 0.05$

Arabidopsis (Vannini et al. 2004), *Osteospermum ecklonis* (Laura et al. 2010), and apple (Pasquali et al. 2008) that overexpressed *Osmyb4*. On the other hand, the findings of Vannini et al. (2007) were in accordance with our data. The authors compared the responses of *Arabidopsis* and tomato and reported that freezing tolerance was not improved in transgenic tomato overexpressing *Osmyb4*. They suggested that different selective pressures in response to the different environments colonized by the two species may have led to their different behaviors upon exposure to stress. Tomato is a tropical plant, and selection pressures could have caused the loss of target genes associated with MYB4 function in response to low temperatures. Another research group also demonstrated that the tomato CBF regulon involved in freezing tolerance is much smaller in size and potentially less diverse in function compared to *Arabidopsis* (Zhang et al. 2004). The comparable responses of potato and tomato upon exposure to freezing temperatures suggest that the target genes of MYB4 in tomato and potato that impact on the response to cold may have evolved in the same direction. Loss of target genes in the response pathway to cold may have resulted in frost sensitivity in both members of the Solanaceae family. This closely related evolution should be investigated in further studies.

Effect of ectopic *Osmyb4* expression on pigment and sugar contents

Total sugar, chlorophyll, total carotenoid, and anthocyanin contents in the WT and transgenic lines were determined to elucidate a possible involvement of MYB4 TF in the accumulation of sugar and different pigment groups in potato. The pigment and sugar contents of only two transgenic lines (S2 and M48) that exhibited high salinity tolerance were analyzed and compared with those of the WT. There were no significant ($P < 0.05$) differences in chlorophyll and carotenoid contents between M48 (pCOR line) and the WT, whereas the carotenoid content in S2 (pCaMV line) was significantly higher (Table 5). The high carotenoid content in the S2 overexpressor line suggests that the accumulation of this pigment may be dependent on the expression level of the *Osmyb4* gene. The anthocyanin contents were not significantly altered in the transgenic lines. Many MYB proteins are thought to play key roles in the regulation of anthocyanin biosynthesis in plants (Chen et al. 2010; Dubos et al. 2010). A myb-related transcription factor gene of the anthocyanin biosynthetic pathway, *VlmybA2*, from the Kyoho grape (*Vitis labruscana*) was shown to induce anthocyanin production in transformed tobacco and *Arabidopsis* (Geekiyanage et al. 2007). Certain MYB proteins can also be negative regulators, such as MdMYB6, which is an R2R3-type MYB transcription factor from apples (*Malus domestica*). Transgenic *Arabidopsis* lines overexpressing *MdMYB6* accumulated less anthocyanin than wild-type plants (Gao et al. 2011). In the present study, the lack of any significant alteration in the anthocyanin contents of the transgenic lines suggests that MYB4 is not involved in the regulation of anthocyanin biosynthesis in potato. Since anthocyanins are stress-responsive compounds in vegetative tissues, determining anthocyanin in the leaves of WT and transgenic plants after exposure to stress may better elucidate the role of MYB4 in the regulation of anthocyanin biosynthesis in potato.

Total sugar contents in the leaves of transgenic potato lines were significantly higher than in the WT (Table 5). This result is in accordance with results obtained in transgenic *Arabidopsis* (Mattana et al. 2005), apple (Pasquali et al. 2008), and tomato (Vannini et al. 2007) overexpressing *Osmyb4*. Transgenic *Arabidopsis*, apple, and tomato plants accumulated higher amounts of sugars than the WT did, both before any stress treatment and upon exposure to stresses such as cold and drought. Plants acclimate to various stresses via the accumulation of sugars, which serve as osmoprotectants during the stress. The increased concentrations of sugars seen in the *Osmyb4* transgenic plants in the present work indicate that MYB4 may be involved in the regulation of sugar metabolism and the activation of some stress-responsive pathways in potato.

In conclusion, the results obtained in this study show that heterologous expression of *Osmyb4* does not exert a negative effect on growth and tuber yield in potato.

Increased carotenoid and sugar contents in the transgenic lines indicate a possible involvement of MYB4 in the regulation of sugar metabolism and the biosynthesis of pigments in potato. Increased tolerance to salinity stress in the S2 and M48 lines suggests a possible role for MYB4 in the salt stress response of potato. Detailed biochemical and gene expression analyses may allow us to more deeply probe the mechanisms underlying salinity stress tolerance in transgenic potato plants expressing *Osmyb4*. Contrary to the results obtained in transgenic *Arabidopsis*, *O. ecklonis*, and apple overexpressing *Osmyb4*, no improvement in freezing tolerance was observed for transgenic potato. The frost sensitivity of transgenic potato suggests that MYB4 is not involved in the regulation of cold-responsive pathways in *Solanum tuberosum*. Comparative analysis of gene expression profiles in WT and transgenic lines may lead to a better understanding of the role of MYB4 in potato. To this end, we have already carried out microarray analysis. A detailed analysis of the microarray data will provide an overall view of the target genes of MYB4 in potato.

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