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Genetic Engineering for Increasing Salinity Tolerance in Tomato

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Abstract: *Agrobacterium* **–** mediated genetic transformation system has been developed for two tomato *(Solanum Lycopersicum)* genotypes. Prior to the establishment of transformation, protocol, explants of cotyledons from the two varieties were cultured to obtain genotype independent *in vitro* regeneration. Healthy multiple shoot regeneration was obtained from explants of cotyledon cultured on MS medium containing 2.0 mg/l BAP and 0.1 mg/l IAA. The maximum root induction from the regenerated shoots was achieved on half the strength of MS medium supplemented with 0.1 mg/l NAA. The *in vitro* grown plantlets were successfully transplanted into soil where they flowered and produced fruits identical to those developed by control plants. Oxo-phytodienoate reductase (OPRs) are enzymes codes for a group of related flavoenzymes. The name is derived from the only member for which the function has been established, i.e. OPR3 that catalyzes the reduction of 12-oxo-phytodienoic acid (OPDA) in the octadecanoid pathway for jasmonic acid (JA) biosynthesis. Transformed cotyledon explants were found to produce multiple shoots on MS containing 2.0 mg/l BAP and 0.1 mg/l IAA. Selection of the transformed shoots was carried out by gradually increasing the concentration of kanamycin to 100 mg/l since kanamycin resistant gene was used for transformation experiments. Shoots that survived under selection pressure were subjected to rooting. Transformed rooted plantlets were transferred to soil. Stable expression of *AtOPR1* gene was detected in the various tissues from putatively transformed plantlets. The transgenic plants exhibited an enhanced level of resistance to salt stress during seed germination.

Key words: tomato, Agrobacterium-mediated transformation, genotype, growth regulators, *AtOPR1*, salt tolerance.

INTRODUCTION

One of the major targets for biotechnology in Egypt is the production of transgenic plants conferring resistance to abiotic stress such as non-favorable environmental conditions including soil salinity, drought and high temperature. These abiotic constraints are major agricultural problems leading to deleterious yield losses in a large variety of economically important crops in Egypt.

Salinity is the most important abiotic factor limiting plant growth and crop productivity worldwide (Bray *et al*., 2000). Salinity is becoming particularly widespread in many regions, and may lead to the loss of more than 50% of all arable lands by the year 2050 (Wang *et al*., 2003). Arable land acreage is limited in Egypt due to the lack of water needed for irrigation. Egypt is adopting furrow irrigation-systems and is also expanding in cultivating the desert. Salinity, water shortage and low water quality are the main problems for agricultural production under such circumstances. The north coast of Egypt is comprised of marginal land. The available irrigation water has a relatively high salt content. Other areas in Egypt and many other areas in the arid zones of the world are experiencing similar problems of increased salinity of soils, and/or irrigation water. The ability of plants to tolerate excess salts in the rhizosphere is of considerable importance in the arid and semi-arid regions where salinization of soils usually prevails.

Tomato (*Solanum lycopersicum*) is the most important vegetable crop grown in Egypt (Ramadan and Adam, 2007). Egypt's population grows with an annual birth rate of 2.7%. The number of Egypt's population by the year 2025 will rise to 110 million. The gap between future supply and demand in tomato makes it imperative to increase cultivation in the areas with sub optimal conditions, such as water deficit and salinity.

Genetic engineering offers the opportunity to introduce new genes into tomato germplasm. Since the first report of tomato transformation (McCormick *et al*., 1986), there have been numerous publications of genetic manipulation on several cultivars of tomato (Chi and Phillips, 1987; Fillatti *et al*., 1987; Fischhoff *et al*., 1987; Delnnay *et al*., 1989; Van Roekel *et al*., 1993; Agharbaoui *et al*., 1995; Frary and Earle, 1996; Ling *et al*., 1998; Tabaeizadeh *et al*., 1999; Vidya *et al*., 2000; Hu and Phillips, 2001; Shahriari *et al*., 2006; Sarker *et al*., 2009). However, the transformation of the tomato using *Agrbacterium tumefaciens* is highly dependent on genotype. There are fewer articles on transformation directed at increasing the salt tolerance of tomato (Rhodes and Hanson, 1993; Arrillaga *et al*., 1998; Gisbert *et al*., 2000; Moghaieb *et al*., 2000; Rus *et al*., 2001; Zhang and Blumwald, 2001; Jia *et al*., 2002; Flowers, 2004; Cuartero *et al*., 2006; Raja *et al*., 2012).

In many species, genes encoding 12-oxo-phytodienoic acid reductase (OPRs) comprise a multigene family. In the *Arabidopsis* and tomato genomes, three OPRs have been identified (Biesgen and Weiler, 1999; Strassner

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et al., 2002). In *Arabidopsis,* AtOPRl and AtOPR2 are classified into the OPRI group where they preferentially catalyze the reduction of *cix-(-)* OPDA over *cis-(+)* OPDA, the natural precursor of jasmonic acid (JA). As such, they are not enzymes associated with the JA biosynthctic pathway (Schaller *et al*., 1998). The *in vivo* substrate of OPRI has not been identified. OPRs within a species are differentially regulated by various environmental stimuli. In *Arabidopsis,* stress treatment such as wounding and cold resulted in transient changes at the *AtOPRl* and *AtOPK2* mRNA level (Biesgen and Weiler, 1999), In monocot plants, OsOPRl has been characterized at the biochemical and molecular level. The *OsOPRl* transcript is up-regulated under a broad range of stress conditions (Agrawal *et al*., 2003). Recent research has shown that it is now possible to use transgenic approaches to increase salinity tolerance using *AtOPR1* (Gu *et al*., 2008).

The goal of this search is finding out new possibilities to improve the conditions for genetic engineering of tomato, and identifying suitable tomato genotype to such modifications through setting up an *in vitro* regeneration procedure of cotyledon explants, as well as to investigate the role that AtOPR1 plays in plant response to salt stress conditions during seed germination.

MATERIALS AND METHODS

Plant Material:

Mature seeds of two tomato genotypes were used to raise seedlings for the present study. Seeds of the genotype Strain B and Peto 82 were obtained from the Preservation Germplasm Laboratory of the Dpartment of Horticulture, Faculty of Agriculture, Benha University.

Establishment of Aseptic Plant Cultures:

For establishing aseptic cultures of tomato growing *in vitro*, dry mature seeds were surface sterilized with sodium hypochlorite a common disinfectant for surfaces of plant tissue. Seeds of the tomato genotypes were immersed in a 2.5% sodium hypochlorite for 10 min which is present in commercial bleach solutions (Clorox). Then they were rinsed five times with sterile distilled water for 10 min each. During immersion and rinsing the solution was stirred on a shaker at 200 rpm under the laminar air flow hood. The sterilized seeds were placed into sterile tissue culture jars (30 seeds/jar) containing a half concentrated basal MS medium (Murashige and Skoog 1962) supplemented with B5 vitamins (Gamborg *et al.,* 1968), 3.0% sucrose and solidified with 0.7% Oxoid-Agar (Fig.1a). The medium was adjusted to pH 5.8 before autoclaving at 121°C and 1.2 kg cm-2 to 1.3 kg cm-2 pressure for 20 min. All cultures were incubated at 25°C ± 1°C in the dark.

Regeneration of Plants From Cotyledons:

Cotyledons from ten days old seedlings were excised and used as explants (Fig. 1b). Each cotyledon was cut into three pieces with the part closest to the cotyledon termed "basal", the next "sub-apical" and the part at the tip of the cotyledon "apical". Only the "sub-apical" explants were used for the experiments (Fig.1c). Explants induction media for all experiments contained 7 g/l agar and 30 g/l sucrose. Explants were sub-cultured every 2 weeks. The MS media were adjusted to 5.8 pH prior autoclaving at 121 ºC for 25 minutes. For induction and production of multiple shoots from cotyledon explants**,** MS medium supplemented with different concentrations and combinations of BAP, IAA and zeatin were used. For root formation from the cut ends of regenerated excised shoots, half strengths of MS medium supplemented with 0.1 mg/l IAA was used. All media combinations contained 3% sucrose solidified with a 0.8% agar and with a pH of 5.8, adjusted before autoclaving. Cultures were maintained under a regime of 16 h photoperiod at 25±1ºC. Following the development of sufficient roots, plantlets were transferred to small pots containing sterilized soil. These plantlets were acclimated and then transferred to the greenhouse and maintained their till flowering and fruiting. The experiment was carried out with five replicates.

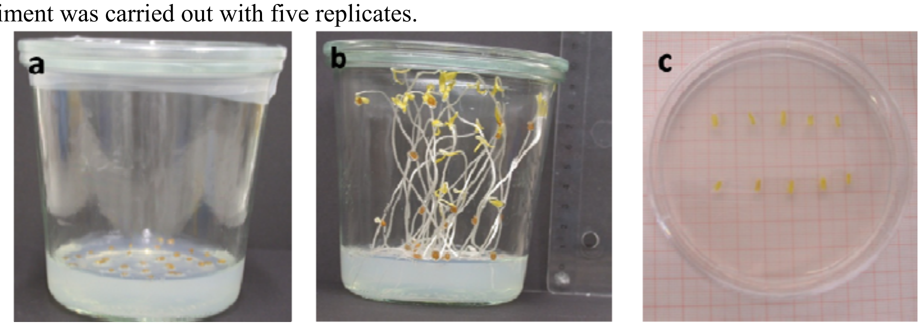


Fig. 1: Explants of cotyledons used for *in vitro* regeneration. (a) Sterilized tomato seeds placed into MS medium. (b) Germinated seeds of cv. Strain B ten days after incubated in the dark. (c) Cotyledon explants used for regeneration and transformation experiments.

Genetic Transformation System:

Agrobacterium tumefaciens strain GV3101 with the binary plasmid pBI121 was used for transformation. It contains a selectable marker gene *nptII* encoding the enzyme neomycin phosphotransferase conferring kanamycin resistance(Pridmore 1987) and *AtOPR1* gene. *A. tumefaciens* strain was provided by Dr. A. Schaller, Institute of plant Physiology and Biotechnology, University of Hohenheim, Germany. Fifty ml of liquid YEB (yeast extract broth) containing 50 mg/l kanamycin were inoculated with *Agrobacterium* from a fresh bacterial plate and grown at 200 rpm on a rotary shaker at 28ºC for 16 h. The culture was subsequently spun at 5000 rpm at 20ºC for 10 min in a centrifuge and the pellet resuspended in 10 ml liquid MS maintaining the optical density 1.0. The explants were prepared with a scalpel keeping the cotyledon explants in the *Agrobacterium* suspension. The cut explants were incubated in the *Agrobacterium* suspension in a small petri plate for an additional period of 30 min. They were then blotted dry on a sterilized Whatman filter paper and cocultured in petri plates on MS medium with 2.0 mg/l BAP and 0.1 mg/l NAA (best regeneration medium selected during regeneration experiments) for three days in the dark. Following co-culture the explants were washed several washed times in liquid Ms medium with gentle shaking until no opaque suspension was seen. The infected explants were finally dried with a sterile Whatman filter paper and placed on the regeneration medium. The infected explants were then placed in the growth room for regeneration under 16/8 hours light/dark cycle at 25 ± 1ºC. To eliminate non-transformed tissues, the regenerating explants were subcultured on a fresh regeneration medium initially with 35mg/l kanamycin after two weeks. The concentrations of selection antibotic was increased with each sub-culture at two weeks intervals up to 100mg/l kanamycin. During each subculture the dead and deep brown tissues were discarded and green shoots and shoot buds were sub- cultured on fresh medium containing the next higher concentration of kanamycin.

Acclimatization and Field Transfer:

Regenrated putative transgenic shoots (approximately 12 cm long) with well developed leaves and roots were transferred to pots containing soil with high relative humidity and maintained in the growth room under 12 h light photoperiod at 25ºC. Plantlets were watered twice a week with water. Then, after two weeks, plantlets were transferred into greenhouse.

Molecular Analysis:

For the examination of *AtOPR1* gene, RNA was isolated from young seedlings (10 days after germination and the cDNA synthesized as described by Gu *et al*., 2008). Reverse transcription PCR (RT-PCR) is used for mRNA detection and quantification. The technique consists of two parts: the synthesis of cDNA from RNA by reverse transcription and the amplification of a specific cDNA by PCR. The RT-PCR reaction was conducted using the RevertAid™ Minus First Strand cDNA Synthesis kit following the manufacturer's instructions. In brief, a reaction mixture was prepared containing 5 µg isolated total RNA, five units RNase free DNase and DEPC-treated water up to 11 µl was prepared. The reaction was incubated at 37 °C for 45 min, and then at 70 °C for 15 min, then chilled on ice. 1.0 µl oligo-dT (500µg/ml) was then added and the mixture was incubated for 5 min at 70 °C. To this mixture 4 µl of 5x Reaction Buffer, 2 µl of 0.1 M DTT and 2 µl of 10 mM dNTPs were added and the reaction was incubated for 5 minutes at 37 °C. One µl of RevertAid™ H Minus M-MuLV Reverse Transcriptase (200u/µl) was added and the mixture was incubated at 42 °C for 60 minutes, followed by 70 °C for 10 minutes. Finally the reaction was diluted with sterile water and 4 µl were used in subsequent PCR reactions. The PCR reaction was typically carried out in a 25 µl reaction volume with the following constituents: 10-50 ng template DNA, 5 pmole forward primer (5´TCATCAACAGTTTTTACAAAAGAAATG-3´), 5 pmole reverse primer (5´AACACACTACATTACATTATTGATAACA-3´), 0.2 mM dNTPs, 5 µl of 5x *Taq*-buffer, 2 U *Taq*-polymerase and H2O up to 25 µl. The amplification conditions were done in a PCR thermocycler using 94º C for 3 min; 10 cycles at 94º C for 30 s, 60º C (-0.5º C per cycle) for 30 s, 72º C for 45 s; 25 cycles at 94º C for 30 s, 72º C for 45 s. The program was terminated by a final extension step at 72º C for 10 min. The amplification products were analyzed by electrophoresis on 1% agarose gels and visualized with ethidium bromide using standard procedures (Sambrook *et al*., 1989).

Salt Tolerance Assay:

For germination assays, 100 seeds from homozygous transgenic plants and wild-type plants were sterilized and planted on MS medium supplemented with 30 g/l sucrose, 8 g/l agar and various concentrations of NaCI (0, 100, 150 and 200 mM). Cultures were incubated in the tissue culture growth chamber (16 h light/8 h dark) at 25 ± 1ºC. The germinated seeds (emergence of radicals) were counted after one week. For the early seedling growth assay, 50 seeds were planted on the same medium and culture conditions. Seedlings with green cotyledons were counted after 10 days of incubation. This assay was conducted with three replications.

Experimental Design and Statistical Analysis:

Experiments were arranged in a completely randomized block design with five replications. Data were estimated as the mean and its standard error of the different traits. The Calculations were done using Microsoft Excel 2010 program.

RESULTS AND DISCUSSION

Regeneration of Plants from Cotyledons:

The success in tomato transformation could be largely affected by the genotype and plant growth regulators used in culture medium. In this investigation responses of all different combinations and concentrations of growth regulators supplemented with MS medium for the induction of multiple shoots from cotyledon explants of two elite cultivars, which are widely used for commercial purpose in Egypt are presented in Table 1. Explants of cotyledon showed different responses in the presence of various growth regulators. The maximum shoot regeneration was observed on MS media supplemented with 1 mg/l Zeatin and 0.1 NAA of genotype Strain B, but it was observed that 2 mg/l BAP and 0.1 NAA had positive effect towards multiple shoot regeneration of genotype Peto 82 (Table 1). It was found that number of shoots per cotyledon explant increase with the increase of BAP concentration but not with the increase of zeatin concentration (combination with NAA). Many researches have previously reported that Zeatin stimulates the organogenesis of tomato cotyledons (Frary and Earle, 1996; Costa *et al*., 2000; Dorri and Altmann, 2001; Prematilake *et al*., 2002; Park *et al*., 2003; Shahriari *et al*., 2006) which quite agree with our results obtained on BAP. BAP is commonly available and less costly cytokinin than zeatin and sufficient shoots were obtained in the present study using BAP as a cytokine supplemented for *in vitro* regeneration.

It seems that all steps of the regeneration process were cultivar-dependent. The optimal medium for shoot regeneration is quite dependent on cultivars as reported by few researchers (Park *et al*., 2003; Shahriari *et al*., 2006).

Cotyledons were able to produce shoots within 2-3 weeks and by the end of 4th week, the mean number of shoots per cotyledon explant was scored and used as a regeneration efficiency for all two genotypes (Table 1). These results were similar to the results obtained by Moghaieb *et al*., 2006. Considering the regeneration ability the combination of 2.0 mg/l BAP and 0.1 mg/l NAA was used for shoot regeneration (Fig. 2a). Shoots (3 cm) were rooted using half strength of agar solidified MS medium supplemented with 0.1 mg/l IAA (Fig. 2b). Results were in agreement with other researchers (Oktem *et al*., 1999; Romero *et al*., 2001; Lino *et al*., 2004; Sarker *et al*., 2009).

Rooted plantlets were transferred to soil where they successfully acclimatized (Fig. 2c) and produced flower and subsequently fruits with viable seeds (Fig. 2d). From the above findings it can be concluded that this regeneration protocol developed in this study is relatively simple, reproducible and genotype independent.

Growth regulators mg/l \ Cultivars			Strain B		Peto 82	
BAP	Zeatin	NAA	No. of shoots/explant	Time of shoot differentiation (days)	No. of shoots/explant	Time of shoot differentiation (days)
0.5	-	0.1	2.2 ± 0.4	21	4.5± 0.6	18
1	-	0.1	4.8 ± 0.6	18	10.5 ± 1.2	15
2	-	0.1	6.2 ± 0.8	15	12.7 ± 1.3	14
-	0.5	0.1	5.4 ± 0.5	21	4.2 ± 0.7	21
-	1	0.1	9.8 ± 1.0	14	6.5 ± 1.3	15
-	2	0.1	7.0 ± 1.2	12	3.5 ± 0.6	12

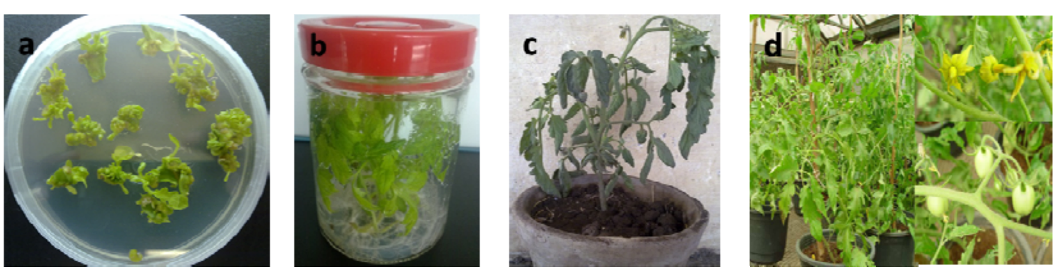


Fig. 2: *In vitro* regeneration of cotyledons of genotype Peto 82. (a) Initiation of multiple shoot on MS medium with 2 mg/l BAP and 0.1 mg/NAA. (b) Root formation from excised shoots cultured on half strength of agar solidified MS medium supplemented with 0.1 mg/l IAA. (c) Acclimatization of regenerated tomato plantlet using soil. (d) Normal regenerated tomato plants in the greenhouse.

Genetic Transformation System:

The *Agrobacterium tumefaciens* strain GV3101 used in this study has *AtOPR1* gene and *nptII* gene and this gene confers kanamycin resistance to the transformed cells. Therefore, selection of the transformants was carried out using various concentrations of kanamycin. It was observed that co-cultivated explants of cotyledon, even in the presence of lower concentrations of kanamycin in regeneration medium, failed to regenerate and consequently died. This observation was similar to the results obtained by Prematilake *et al.*, 2002; Sarker *et al.*, 2009. therefore, selection pressure was not applied immediately after co-cultivation; instead, co-cultivated explants were first allowed to regenerate in regeneration media without any selective agents. After two weeks the infected cotyledon explants showing very small shoots and shoot buds were subjected to selection pressure. All control shoots died in the selection medium with 200mg/l kanamycin. In this investigation, a lower concentration of kanamycin (35 mg/l) was applied in the initial selection medium and selection pressure was increased gradually in subsequent subcultures. For selection, 50-100mg/l kanamycin was reported to be suitable in obtaining transformed tomato shoot (Ling *et al.,* 1998, Tabaeizadeh *et al.*,1999, Cortina and Culianez- Mica, 2004). In the present study, the shoots that survived in presence of 50 mg/ l kanamycin were subjected to higher selection pressure with 100mg/l kanamycin to obtain transformants. Putative transgenic shoot of cv. Strain B continued to grow and produced plants with a well – developed root on MS medium supplemented with 0.1 mg/l IAA. On the other hand, none of the transformed cotyledons explants of cv. Peto 82 grew on the selective medium with 50 mg/ kanamycin.

Detection of AtOPR1 Expression Patterns by RT-PCR:

To determine the organ-specific expression pattern of the *AtOPR1* gene, RT-PCR was performed on RNA isolated from roots, Stems and leaves, (Fig. 3). It revealed that *AtOPR1* transcript was found to be abundant in leaves, stems and roots, but the expression was stronger in stems and roots, as compared to leaves. Similar results were reported by Gu *et al*., 2008.

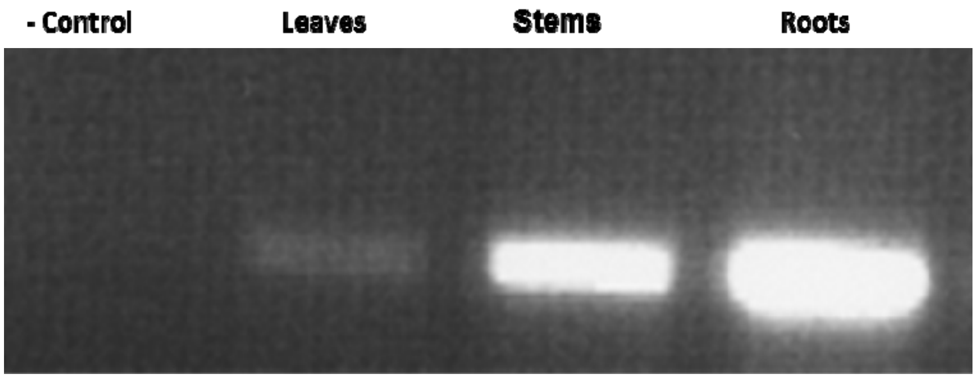
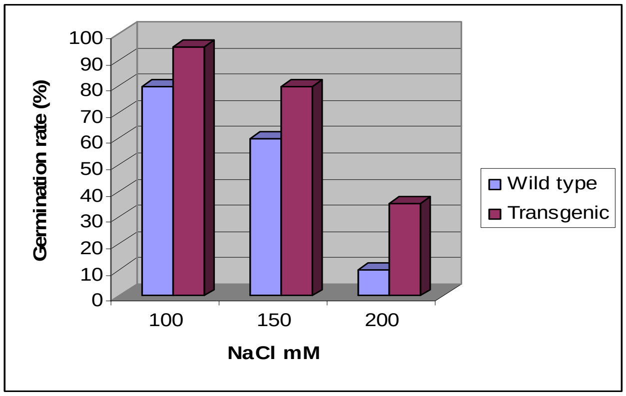


Fig. 3: AtOPR1 tissue-specific and growth stage-based gene expression.

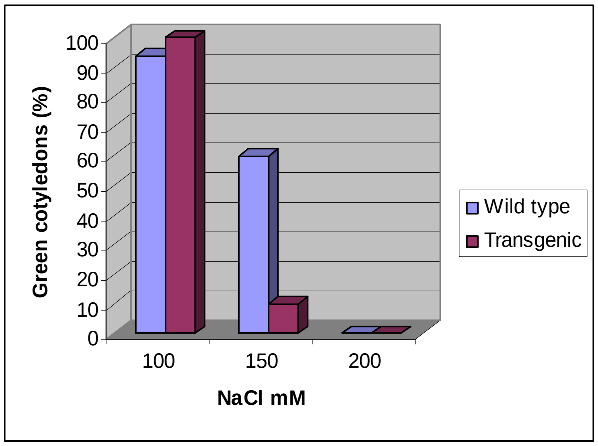
AtOPR1 mRNA levels as measured by RT-PCR. RNAs were extracted from various *tomato* tissues as indicated.- Control is a negative PCR control without cDNA template.

Expression of AtOPRI Enhanced the Tolerance of Transgenic Tomato to NaCI Stress:

Salt tolerance of transgenic plants (T1 generation) was evaluated by germinating seeds (T1) and the wild type on MS agar medium supplemented with 50, 100, 150 and 200 mM Nacl. The germination rate of transgenic tomato seeds was significantly higher than that of non-transgenic plants subject to various concentrations of NaCI. As shown in Fig. 4, T1 of cv. Strain B had higher germination rates 95% than WT (80%) at low NaCl concentrations (100 mM). The mean germination rate was 80% for transgenic plants grown on MS medium containing 150 mM NaCl, which was significantly higher than that of the wild type (60%). Although the germination rate decreased substantially with increases in salt concentration 200 mM in both the transgenic and wild-type seeds, a significant difference remained between them (Fig. 4a). The percentage of seedlings with green cotyledons in transgenic plants was also significantly higher than that of wild type on MS medium containing 150 mM NaCl. When the concentration of NaCl was increased to 200 mM, no seedlings with green cotyledons could be observed (Fig. 4B), which suggests that expression of *AtOPRl* also conferred an increased tolerance to salt stress in transgenic tomato plants. Most studies on JA have focused on plant responses to wounding and insect attacks, and might act as a regulator in plant response to pathogens (Zhang *et al*., 2005). Current results may provide new insight into the role of the OPRI group in the osmotic stress and salt signaling pathway, which was also supported by previous reports that the expression *OsOPRl* in rice was up-regulated by drought (Agrawal *et al*., 2003).



(a)



(b)

Fig. 4: Effect of different concentrations of NaCl on seed germination of wild and transgenic (T1) tomato. (a) Seed germination rate. (b) Seedling emergency rate with green cotyledons.

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