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ORIGINAL PAPER

# Generation of marker free salt tolerant transgenic plants of *Arabidopsis thaliana* using the gly I gene and cre gene under inducible promoters

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**Abstract** Despite the advances in transgenesis, transformation technologies still rely on the introduction of a selectable marker gene to identify cells and tissues that have integrated the gene of interest in their genome. The continuous presence of the marker genes in the transgenics is often controversial as it can potentially have multiple undesirable impacts. The present study employed the self-excising Cre-*loxP* system to generate marker-free *Arabidopsis thaliana* expressing the agronomically important *glyoxalase I* (*glyI*) gene from *Brassica juncea* to confer salt stress tolerance. A binary vector was constructed wherein the salt-inducible rd29A promoter was used to drive the expression of the *glyI* gene and the transformants of *A. thaliana* were recovered using kanamycin resistance as the selectable marker. The *neomycin phosphotransferase II* (*nptII*) gene was flanked by the *loxP* sites followed by the introduction of a heat-inducible Cre-recombinase in between the *loxP* sites. The kanamycin-resistant transgenic lines of *A. thaliana* using this vector showed an ability to withstand stress imposed by 150 mM NaCl. The exposure of the T2 transgenic lines to a mild heat shock (37°C) resulted in the recovery of salt-tolerant, kanamycin-sensitive T3 progeny. Molecular analyses of the T3 transgenic lines following the heat shock treatment confirmed the excision of the *nptII* gene and the completion of their life cycle in the presence of 150 mM NaCl-induced stress.

**Keywords** *Arabidopsis thaliana* · hsp-*cre* · *npt-lox* · *Bj glyoxalase I* gene · rd29A promoter · Salt stress tolerance

**Abbreviations**

| | |
|---|---|
| ABA | Abscisic acid |
| ABRE | ABA- responsive element |
| Cre | Cre-recombinase |
| DRE | Drought responsive element |
| gly I | Glyoxalase I |
| hsp | Heat shock promoter |
| MS | Murashige and Skoog |
| SPB | Sodium Phosphate Buffer |
| GSH | Reduced glutathione |
| PMSF | Phenylmethylsulfonylfluoride |
| PVPP | Polyvinyl polypyrrolidone |

## Introduction

Glyoxalase I (EC 4.4.1.5, lactoylglutathione lyase) and glyoxalase II (EC 3.1.2.6, hydroxacylglutathione hydrolase) constitute the glyoxalase system. In a two

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step reaction, these enzymes act using glutathione as a cofactor to coordinately convert cytotoxic methylglyoxal and other 2-oxoaldehydes to their 2-hydroxyacids (Thornalley 1990) as follows

$$\text{Methylglyoxal} + \text{Glutathione} \leftrightarrow \text{Hemithioacetal} \overset{glyoxalase\ I}{\leftrightarrow} \text{S-D lactoylglutathione}$$

$$\text{S-D lactoylglutathione} \overset{glyoxalase\ II}{\leftrightarrow} \text{Lactate} + \text{Glutathione}$$

Glyoxalase I first catalyses the formation of S-D-lactoylglutathione from hemithioacetal, which is formed by a non-enzymatic reaction between reduced glutathione (GSH) and methylglyoxal. Glyoxalase II catalyzes the hydrolysis of this S-D-lactoylglutathione to D-lactate with the regeneration of GSH in the second step (Uotila 1989). The primary physiological function of the glyoxalase system appears to be the detoxification of methylglyoxal, which is mainly synthesized as a byproduct of carbohydrate metabolism. Besides this, the glyoxalase system also increases the level of free reduced glutathione which is essential for scavenging of toxic reactive oxygen species (such as $H_2O_2$) and organic peroxides) that are increased in plants under stress conditions and in the maintenance of other antioxidants such as ascorbates and tocopherols (Alscher 1989). The involvement of the glyoxalase system in plants under stress conditions was first observed by Esparteo et al. (1995) where the glyoxalase I activity was shown to be upregulated under abiotic stresses. Overexpression of the *glyoxalase I* (*gly1*) gene under the control of the constitutive CaMV35S promoter in tobacco and rice has been shown to impart tolerance to salt, drought, and heavy metal stress (Veena et al. 1999; Singla-Pareek et al. 2003, 2006). Similarly, CaMV35S–mediated constitutive overexpression of *glyoxalase II*, the other gene of this system, has been shown to confer salt tolerance in tobacco (Singla-Pareek et al. 2003) and rice (Singla-Pareek et al. 2008). Since both the enzymes act in the same pathway, overexpression of either of these enzymes automatically shifts the enzyme catalyzed reaction in the forward direction that ultimately leads to increased release of reduced glutathione that ultimately detoxifies reactive oxygen species. This might result in a similar stress tolerant phenotype in the plants overexpressing either of the enzymes. However, constitutive overexpression of the transgene may compete for the building blocks and machinery needed for RNA and protein synthesis under stress-free conditions. Hence we constructed and analyzed plants with transgene expression driven by a stress-inducible promoter, rd29A, so that the specific mRNA and proteins required for stress alleviation are only produced under stress conditions. It has been reported that the rd29A and rd29B genes are induced under conditions of high temperature, high salt or upon treatment with exogenous abscisic acid (Yamaguchi-Shinozaki and Shinozaki 1993a, 1994). The sequence analysis of rd29A promoter showed the presence of drought-responsive element (DRE) and ABA-responsive element (ABRE). The 9 bp DRE element is involved in the first rapid response of rd29A to conditions of dehydration or high salt. ABA has also been found to be produced in plants under stress.

Selectable marker genes (SMGs) are used in nearly all plant transformation experiments and do not serve any purpose after the gene of interest is established. Besides precluding its reuse for future transformation experiments, the continuous presence of the SMG also raises issues of ecological concerns (Hill and Sendashonga 2006). It is, therefore, desirable to remove the selectable marker gene after it has served its crucial role in selection. There are several approaches for the removal of the marker gene like the simultaneous delivery of two T-DNA elements, one having the marker gene and the other having the gene of interest, as used by (Park et al. 2004; Matthews et al. 2001; Chen et al. 2005), transposition mediated repositioning and subsequent elimination of marker genes (Goldsbrough et al. 1993), use of homologous recombination (Iamtham and Day 2000) and the Cre-*loxP* recombination system (Dale and Ow 1991; Russell et al. 1992; Hoa et al. 2002). The Cre-*loxP* recombination system is often used due to its simplicity because apart from the 38 kDa Cre recombinase and the 34 bp *loxP* sites, no other factor is required for recombination to occur

(Sternberg and Hamilton 1981; Stemberg et al. 1986). The *cre* gene for producing the Cre recombinase can be introduced into the *loxP* background by crossing plants harboring the *loxP* sequences with the plants expressing the recombinase gene or by sequential transformation of a plant with *loxP* and *cre* bearing constructs, respectively. Recently, various approaches were investigated to reproducibly obtain optimum CRE activity (Marjanac et al. 2008). An exciting area that deserves attention is the use of site-specific recombinases under the control of inducible promoters to excise SMGs after transgenic plants have been recovered (Hoff et al. 2001; Cuellar et al. 2006). Based on this premise, the *cre* gene under a heat shock inducible promoter (hsp) was cloned in a binary vector containing the *glyI* gene so that the *cre* gene, the *lox*P sequences and the *glyI* gene can be transferred to the target species. *Arabidopsis thaliana* was transformed with this novel construct to obtain salt tolerant kanamycin resistant transformants which upon exposure to a mild heat shock resulted in the recovery of marker-free salt tolerant transgenics. This could be used subsequently for obtaining abiotic stress tolerant marker-free transgenics of agronomically important crops.

## Materials and methods

### Construction of self-excising plant transformation vector

*Escherichia coli* strain DH5α was used as the host for recombinant vector constructions. The binary vector pCAMBIA 2301 (pC2301) which had the *nptII* (neomycin phosphotransferase) gene coding for kanamycin resistance as the plant selectable marker and *gus* (*β*-glucuronidase) as the reporter gene, both driven by the CaMV35S promoter, was chosen as the basic vector for this study. Two pairs of complementary single-stranded oligonucleotides (containing the *loxP* sites) were chemically synthesized (Qiagen Inc.) in such a manner that each pair, when annealed, would result in double stranded *loxP* oligonucleotides having *Eco*RI and *Xho*I overhangs and the restriction sites used for cloning would be preserved after their ligation into the parent vector. These *loxP* oligos were independently cloned into pC2301 after digestion with *Eco*RI and *Xho*I. Thus, the 34 bp *loxP* sequence was introduced both upstream of the 35S promoter (at *Eco*RI restriction site) and also between the *nptII* coding region and the 35S terminator (at *Xho*I restriction site). The positive clones were confirmed by restriction digestion and sequencing. The resultant vector had the *nptII* gene flanked on both the sides by the *loxP* sites in direct orientation and was denoted as p*npt-lox*.

A 950 bp fragment of rd29A promoter (Yamaguchi-Shinozaki and Shinozaki 1993b) was amplified from *A. thaliana* cv. Columbia genomic DNA with primers having *Xba*I and *Nco*I overhangs (5′-GAGC TCTAGATGCAATTCAATCAAACTG-3′ and 5′-TGATCCATGGTCCAAAGATTTTTTTCTTTCCA ATAG-3′). The *Brassica juncea glyI* cDNA, cloned in pBI-SI (Veena et al. 1999), was amplified with primers having *Nco*I and *Bst*EII overhangs (TTCTCCATGGCGTCGGAAGCGAAGGAATC-3′ and 5′-TTTTGGTCACCGATAACAACTTATTTAA CTCAACTC-3′). The binary vector p*npt-lox* was digested with *Xba*I and *Bst*EII, which led to the removal of the *gus* reporter gene. A three-fragment ligation was done with the *Xba*I-*Bst*EII fragment of the vector, *Xba*I-*Nco*I fragment of rd29A promoter and *Nco*I-*Bst*EII fragment of the *glyI* gene. This resulted in the construct p*npt-lox* + rd29A-*glyI*, wherein the gene of interest, *glyI* was placed under the control of the rd29A promoter. Finally, a DNA fragment comprising the *cre*-recombinase (hsp-*cre*) driven by the heat-shock inducible promoter was obtained as a PCR product using primers with *Eco*RI and *Bam*HI overhangs (5′-GCCAGAATTCATCGGTTTGAAGATG GCAAGTGTT-3′ and 5′-AATTGGATCCTAATCG CCATCTTCCAGCA-3′) and the pCrox 18 vector (Hoff et al. 2001) as the template. The CaMV35S terminator was obtained as a *Bam*HI-*Eco*RI digest from dsProA (Pooggin et al. 2003). The hsp-*cre* fragment and CaMV35S terminator were introduced as a three-fragment ligation into p*npt-lox* + rd29A-*glyI*, which was linearized using *Eco*RI. The integrity and orientation of the double insert was confirmed by restriction analysis and later by sequencing. This resulted in the vector phsp-*cre* + *npt-lox* + rd29A-*glyI* wherein both the *nptII* and hsp-*cre* were flanked by the *loxP* sites. The resulting vector, phsp-*cre* + *npt-lox* + rd29A-*glyI*, contained the *glyI* gene driven by the rd29A promoter and both the *nptII* and hsp-*cre* were flanked by the *loxP* sites (Fig. 1).

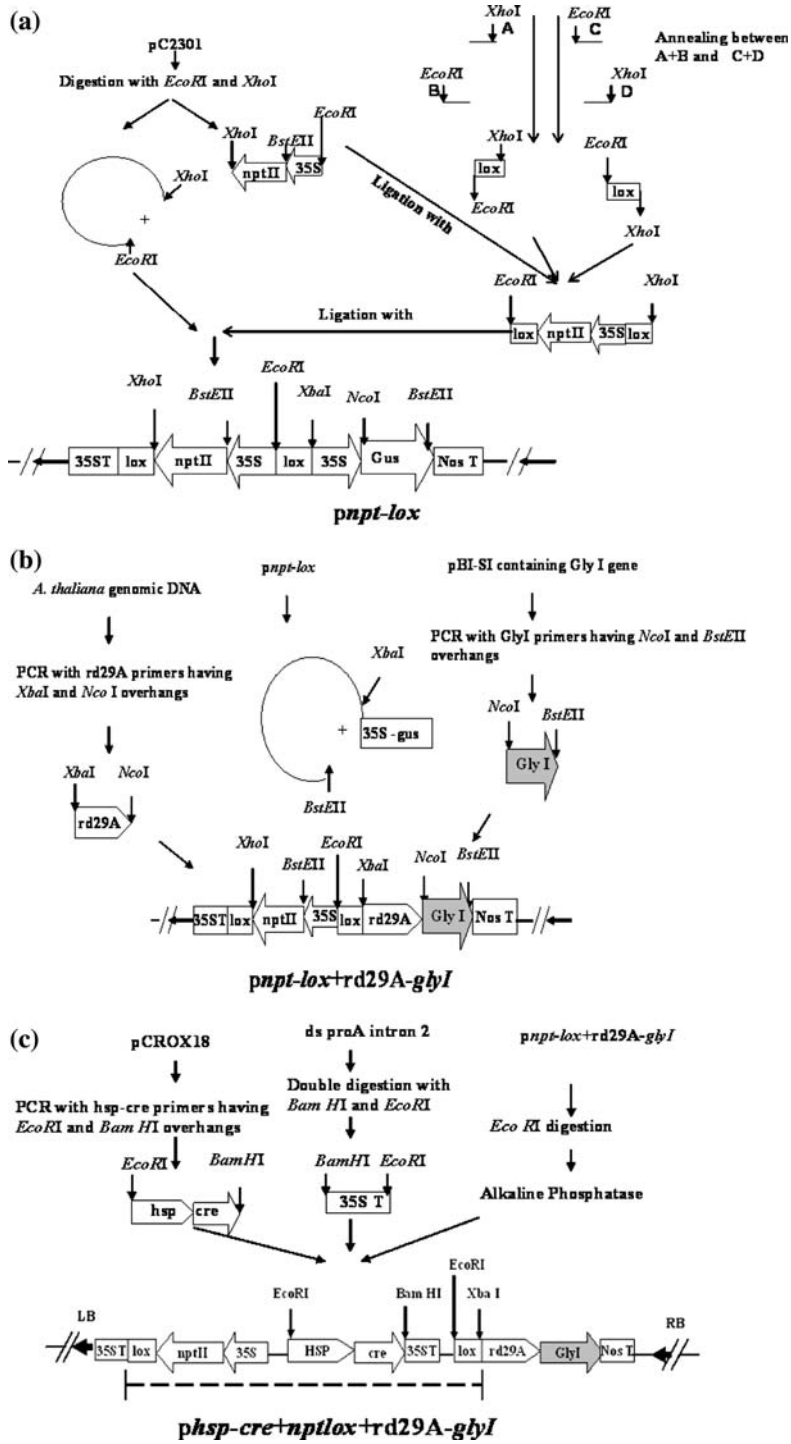


**Fig. 1** Schematic representation of construction of phsp-*cre* + rd29A-*glyI* binary vector used for *A. thaliana* transformation. In the first step two lox sites were introduced in pC2301 to get p*npt-lox* vector (**a**) and then the *glyI* gene was introduced under the control of rd29A promoter at *Xba*I and *BstE*II sites of this vector to develop p*npt-lox* + rd29A-*glyI* vector (**b**) and finally *cre* gene under the control of hsp was introduced in this vector to generate the final phsp-*cre* + rd29A-*glyI* vector (**c**)

The vector phsp-*cre* + *npt-lox* + rd29A-*glyI* was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *A. tumefaciens* was grown on YEB (1.0 g/l yeast extract, beef extract 5 g/l, peptone 5 g/l, sucrose 5 g/l, 0.2 g/l MgSO4, 15 g/l agar) semi-solid medium containing 50 mg/l rifampicin, 25 mg/l gentamycin and 50 mg/l kanamycin. A single bacterial colony was inoculated into

5 ml of liquid YEB containing the same antibiotics and grown overnight at 28°C on a shaker at 200 rpm. A 200 µl aliquot of bacterial suspension was added to 20 ml of YEB liquid medium supplemented with the same antibiotics and grown overnight before using the culture for transformation of *A. thaliana*.

## Plant material and growth conditions

The seeds of *A. thaliana* ecotype Columbia were surface-sterilized by incubation for 1 min in 70% ethanol, 10 min in 2% hypochlorite/0.01% Tween-20 and rinsed four times with sterile water. Seeds were imbibed for 2 days at 4°C before germination in a growth chamber (22°C, 16 h light/8 h dark, 100 µmol $m^{-2}$ $s^{-1}$, 60% relative humidity) on agarified half-strength Murashige–Skoog (MS) medium. Flowering *Arabidopsis* plants were transformed with GV 3101 (phsp-*cre* + *npt-lox* + rd29A-*glyI*) using the floral-dip method (Bechtold et al. 1993).

The T1 seeds from primary transformants were treated as before and selected on half-strength MS medium supplemented with 50 mg $l^{-1}$ kanamycin in magenta boxes. The seedlings were transferred to soil in pots, checked for the presence of the *glyI* and the *nptII* gene by PCR analysis, allowed to self-fertilize and form T2 seeds. The T2 seeds were collected and germinated on selection medium as described above to obtain the T3 progeny.

## PCR and Northern analysis

Total genomic DNA was isolated from the transgenic as well as untransformed control plants by a refined protocol of Murray and Thompson (1980). The 780 bp region of *glyI gene* was amplified using the primers (5′-CGGGGTACCATGGCGTCGGAAGC GAAGG-3′ and 5′-TGCTCTAGCGCTCTCAAGC TGCGTTTCCGGCTG-3′) and the 700 bp *nptII* gene coding region was amplified using the primers (5′-GGAGCGGCGATACCGTAAAGC-3′ and 5′-GAG GCTATTCGGCTATGACTG-3′). The amplification reaction was carried out using a thermal cycler (Techne Inc.) under the following conditions: for *glyI* gene: one cycle of 94°C for 5 min; 29 cycles of 94°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 90 s (extension); a final extension at 72°C for 5 min (one cycle); for *nptII* gene: one cycle of 94°C for 5 min; 29 cycles of 94°C for 60 s (denaturation), 58°C for 30 s (annealing), and 72°C for 45 s (extension); a final extension at 72°C for 5 min (one cycle). The PCR was performed using ca. 100 ng of purified genomic DNA and Taq polymerase (NEB). The amplified products were separated by electrophoresis on a 1% agarose gel (Sambrook et al. 1989) and visualized by ethidium bromide staining.

For Northern blot analysis, the standard protocol of Sambrook et al. (1989) was followed. Total RNA was isolated (Chomczynski and Sachi 1987) separated by formaldehyde gel electrophoresis and probed with $^{32}$P-dCTP-labelled *glyI* cDNA.

## Glyoxalase I assay

Leaves of *A. thaliana* were crushed using liquid nitrogen and mixed thoroughly with the extraction buffer (SPB pH 7.0 also containing 50% glycerol, 16 mM $MgSO_4$, 0.2 mM PMSF, and 0.2% PVPP). The crushed tissue extract was centrifuged twice at 13,000 rpm at 4°C for 30 min so as to obtain the crude protein extract as a clear supernatant. The gly I activity was assayed according to the protocol described by Ramaswamy et al. (1983). The standard enzyme assay mixture comprised 0.1 M SPB pH7.5, 3.5 mM methylglyoxal, 1.7 mM GSH and 16 mM $MgSO_4$ in a final volume of 1 ml. This assay mixture was incubated for 7 min at room temperature prior to addition of crude protein extract (to allow non-enzymatic formation of hemithioacetal from methylglyoxal and GSH). After addition of the protein extract, the gly I activity was measured spectrophotometrically as a function of thioester formation (S-D lactoylglutathione) by measuring the rate of change in absorbance at 240 nm. The molar absorption coefficient of the thioester (SLG) at 240 nm is 3,370 $m^{-1}$ $cm^{-1}$. Three different enzyme extractions were performed per sample for three independent plants of the five T2 generation transformants. The specific activity of enzyme was expressed in units per $mg^{-1}$ of protein.

## Heat induction experiments

The T2 seedlings grown on kanamycin-supplemented MS medium were used for heat induction experiments. In the first phase, the 2-week-old seedlings were incubated at 37°C for 16 h, after which they were allowed to recover at 20°C for 30 h. This was followed by the second phase of heat treatment where

the seedlings were re-incubated at 37°C for 16 h and immediately transferred to half-strength MS medium without kanamycin and maintained at 20°C. After one week they were transferred to soil in the pots, allowed to grow and set seed. The T3 progenies obtained from the heat shocked T2 lines were checked for the marker excision by PCR and Southern blot analyses.

## Results

### Construct designing, transformation and selection of *A. thaliana*

The vector pC2301 was modified such that the *nptII* gene was flanked by the *loxP* sites and the *glyI* gene was cloned downstream of the stress inducible rd29A promoter. The hsp-*cre* fragment from the pCrox18 vector (Hoff et al. 2001) was also introduced in the same vector within the *loxP* sites (see Materials and methods and Fig. 1). The binary vector used in the present investigation was constructed in such a manner that the Cre recombinase induced by heat shock would act at the *loxP* sites excising the hsp-*cre* fragment along with the expression cassette of the marker gene from the transgenic plants which would then contain only the desired gene (*glyI*) of interest. The *glyI* gene driven by the rd29A promoter was presumed to express only when the plants experienced salt stress.

*Arabidopsis thaliana* cv. Columbia was transformed with this modified pC2301 vector and the seeds (T1) were collected and germinated on ½ MS medium containing kanamycin (50 mg $l^{-1}$). The putative transgenic seedlings growing on the selection medium were screened for the presence of the *glyI* and the *nptII* gene, respectively by PCR using the gene specific primers. Forty percent of the putative transgenic plants from different lines gave the expected bands corresponding to ~780 bp for the *glyI* and ~700 bp for the *nptII* gene. No corresponding bands were obtained in the case of untransformed control line. The transgenic lines grew normally and did not show any deleterious effect due to the presence of the *cre* driven by a CaMV35S promoter gene as reported earlier by Coppoolse et al. (2003). When transferred to the soil, they flowered and set seed. The T2 seeds from five of these lines were used for further experiments on salinity stress tolerance and marker excision. Figure 2a shows PCR analysis of the untransformed control plants as well as plants from the five representative lines. The expected bands corresponding to ~780 bp for the *glyI* and ~700 bp for the *nptII* gene are seen in the transgenic lines while they are absent in the untransformed control plant.

### Comparison of salt stress tolerance in transgenic vs. the untransformed control lines

Different transgenic lines were tested for the glyoxalase I enzyme activity (Fig. 2b) and the transgenic line showing maximum enhancement of the activity (Line 3) when compared to untransformed control plant, was checked for salinity tolerance test. Seeds of this transgenic line as well as the untransformed control plants were inoculated in ½ MS medium with 150 mM and 200 mM NaCl, respectively. No germination was observed on 200 mM NaCl in either control or transformed seeds. At 150 mM NaCl only 15% of the untransformed control seeds germinated as against 75% of the T2 transgenic seeds (Fig. 2c). The transgenic plants appeared healthy and normal in morphology, whereas the control plants were stunted and showed slow growth with yellowish leaves.

### Effect of induction of the gly I transgene in *A. thaliana* in response to salt stress

The T2 seeds from the transgenic as well as the untransformed control plants were germinated on ½ MS medium. After one week, 10–15 seedlings from Line 3 were transferred to ½ MS medium containing 50 mM, 100 mM, 150 mM, and 200 mM NaCl, respectively. It was observed that the untransformed control plants grown on 100 and 150 mM NaCl were shorter as compared to the transgenic plants. In ½ MS medium (without the addition of NaCl) all plants flowered at the same time. However, delayed flowering (ca. 10 days) was observed in the untransformed control plants in the presence of even low (50 mM) NaCl concentration in ½ MS medium, in which the flowering in transgenic plants remained unaffected. It was only in the presence of a higher concentration (150 mM) of NaCl in the medium that delayed flowering (ca. 7 days) in the transgenic plants was observed as compared to the untransformed control plants.

Total RNA was isolated from the transgenic as well as the untransformed control plants grown at 0 mM,

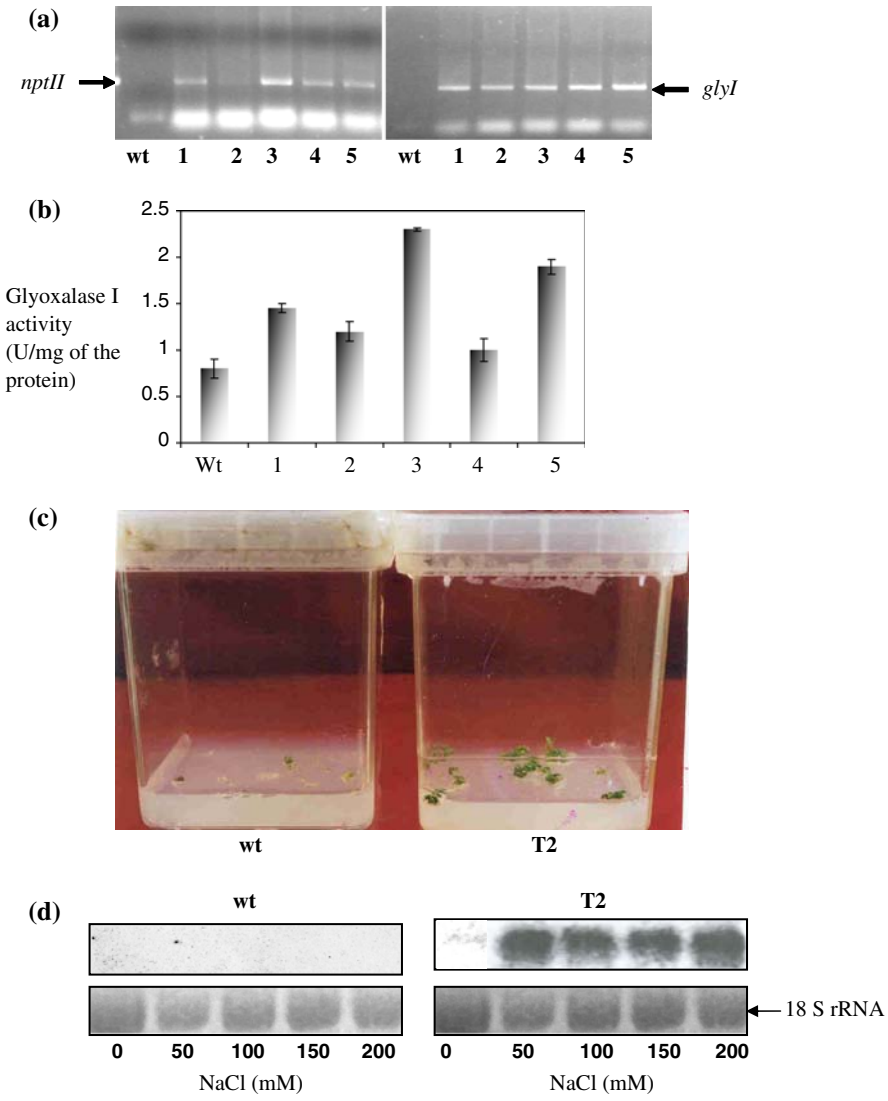


**Fig. 2** Analyses of T2 transgenic lines of *A. thaliana* before the heat shock treatment (**a**) PCR analyses of T2 transgenic lines for the presence of *nptII* gene and the *glyI* gene. (**b**) Glyoxalase I enzyme activities of five independent transgenic lines (1–5) and wild type untransformed control (Wt) plants. The error bars in the graph indicate standard deviation. (**c**) Enhanced germination of T2 transgenic (line 3) vs. untransformed control seeds on ½ MS medium supplemented with 150 mM NaCl. Ten µg of RNA was denatured and electrophoresed through a 1.5% agarose gel containing formaldehyde (7%). Transfer on nylon membrane and blotting was performed according to Sambrook et al. (1989). (**d**) Induction of *glyI* gene expression in transgenic (T2) *A. thaliana* (line 3) exposed to different concentrations of salt (NaCl). The numbers 1, 2, 3, 4, and 5 represent the different transgenic lines, wt represents untransformed control

50 mM, 100 mM, 150 mM, and 200 mM NaCl, blotted and probed with the *glyI* cDNA. No expression of the transgene was seen in the untransformed controls at any of the salt concentrations used (Fig. 2d). Though, hardly any expression was observed in the transgenic plants growing on salt free medium, very good expression of the *glyI* gene was observed on exposure of these plants to salinity stress (Fig. 2d).

## Recovery of marker free salt tolerant transgenic plants

The seeds of the five selected PCR positive T2 transgenic lines were germinated on ½ MS medium with kanamycin (50 mg $l^{-1}$). Fully mature transgenic plants were given heat shock treatment (as described in Material and methods). The heat shock treatment was repeated and the plants were transferred to fresh medium (½ MS without kanamycin). These plants were later transferred to soil where they flowered, self fertilized and set seed. The T3 transgenic seeds, thus obtained, were germinated to obtain the T3 transgenic lines. The T3 transgenic as well as the untransformed control lines were subjected to PCR analysis using the *nptII* and the *glyI* primers, respectively, to check for the excision of the antibiotic resistance marker, *nptII*, gene. Almost, all the transgenic lines which were subjected to heat

shock treatment showed amplification with the *glyI* primers but did not show any amplification with the *nptII* primers (Fig. 3a). This showed that the antibiotic resistance marker gene (*nptII*) had been excised after the heat shock treatment.

To check the expression of the gene of interest, the *glyI*, after the excision of the marker gene, the T3 seeds from the transgenic (Line 3) and untransformed control lines were germinated on ½ MS medium. After one week, the seedlings were transferred to ½ MS medium supplemented with 150 mM NaCl. While the transgenic lines grew normally, flowered and formed seeds, the untransformed control lines showed stunted growth (Fig. 3b). The expression of the *glyI* transgene was checked by northern analysis of the T3 transgenic lines vs. the untransformed control line which confirmed the expression of the gene of interest, the *glyI*, which remained unaffected by the excision of the fragment within the *loxP* sites in response to heat shock (Fig. 3c). Thus, marker free salt tolerant transgenic lines of *A. thaliana* were successfully developed.

## Discussion

Excision of the antibiotic resistance marker gene is desirable for the genetically modified plants to be acceptable to the consumers. Many recombination approaches have been used for the successful deletion of DNA from transgenic plants but the Cre-*loxP* system is one of the best characterized and widely used (Dale and Ow 1991; Russell et al. 1992). Recently, Arumugam et al. (2006) reported the use of Cre-*loxP* system where a passage through in vitro culture of F1 leaf explants resulted in efficient development of marker-free transgenics in F2 generation in *Brassica juncea*. However, no gene of interest was used. In this study, the commonly used binary vector pC2301 was modified such that the plant selection marker gene (*neomycin phosphotransferase II*) and its promoter were flanked by the *loxP* sites. The final vector had the *cre* recombinase gene under the heat shock promoter which was also inserted between the *loxP* sites, thus circumventing the need to co-transform the *cre* gene or wait for another round of transformation. The hsp81-1 promoter was chosen for this study as it has been

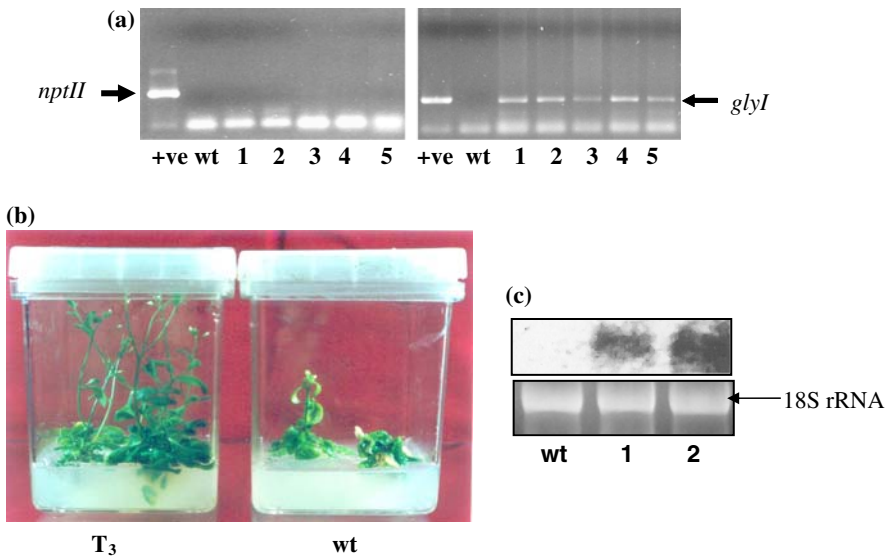


**Fig. 3** Analyses of T3 transgenic lines of *A. thaliana* after the heat shock treatment (**a**) PCR analyses of T3 transgenic lines for the presence of *nptII* gene and the *glyI* gene. (**b**) A representative (T3) transgenic line and the untransformed control line (wt) grown in ½ MS medium supplemented with 150 mM NaCl. (**c**) Northern blot of total RNA of untransformed control and transgenic (T3) *A. thaliana* (line 3) grown in ½ MS medium supplemented with 150 mM NaCl. Ten µg of RNA was denatured and electrophoresed through a 1.5% agarose gel containing formaldehyde (7%). Transfer onto nylon membrane and blotting was performed according to Sambrook et al. (1989). The blot was probed with the *glyI* cDNA. The numbers 1, 2, 3, 4, and 5 represent the different transgenic lines, wt represents untransformed control

reported to be tightly regulated (Hoff et al. 2001) and because of the ease of heat shock treatments to the plants. The gene of interest, the *glyI* gene, driven by the salt inducible promoter rd29A, was present outside the *loxP* sites. This system of auto-excision of the marker gene has several advantages over the crossing strategy. Expression of the *cre* gene for a shorter period of time might overcome the unexpected effects which might arise due to expression for longer duration. Since the selectable marker gene and the *cre* gene can be removed simultaneously, in a single step, while retaining the gene of interest, this methodology offers another advantage in saving time and additional efforts. In this investigation, no deleterious effect of the *cre* gene expression, were observed in the plants. Since the excision of the *nptII* gene occurred after the heat shock treatments while the *glyI* gene was retained, it proves that the approach used in this study was successful for developing agronomically important marker free transgenic plants.

Significant progress has been made towards developing salt stress tolerant plants using various genes (Blumwald et al. 2004). The reports suggest that although abiotic stress is a multigenic trait, salinity stress tolerant plants can be produced by the transfer of a single gene utilizing the transgenic approaches. Overexpression of the *glyoxalase I* gene under the constitutive CaMV35S promoter was shown to impart salt and heavy metal tolerance in transgenic tobacco plants (Veena et al. 1999; Singla-Pareek et al. 2003, 2006. However, in the present investigation, the *glyI* gene driven by a salt inducible promoter has been shown to impart tolerance in transgenic *A. thaliana* plants exposed to salt stress. The *glyI* transcript was observed only in those transgenic lines which were exposed to salinity stress and probed with the transgene.

The transgenic plants were able to cope up better with salt stress, appeared healthier and grew faster as compared to the untransformed control plants under salinity stress. The fact that the salt tolerant transgenic plants developed during this study were also free of the antibiotic resistance marker gene is significant. This strategy if used for the transformation of crop plants will offer at least two major advantages, viz., the expression of the gene of interest under an inducible promoter under salt stress; and a simplified usage of the Cre-*loxP* system where no crossing of the plants having the *cre* gene and the antibiotic resistance gene flanked by *loxP* sites, respectively is required. This not only circumvents the controversies related to marker genes conferring antibiotic resistance, which have practically no use after the transformed plants are established, but also avoids the use of any viral constitutive promoter. Moreover, the foreign DNA (the *glyI* gene) that ultimately remained in the transgenic plants was of plant origin (*B. juncea*). The strategy used in this investigation is similar to that used by Cuellar et al. (2006) for developing antibiotic marker free transgenic potato and by Wang et al. (2005) for tobacco. However, no transgene of agronomic importance was introduced in the transgenic plants. Recently an embryo specific promoter driving the cre gene has been used for generating marker excision in soybean (Li et al. 2007). Other workers (Zuo et al. 2001; Sreekala et al. 2005; Zhang et al. 2003) have used chemically induced autoexcision of selectable markers. The efficiency of the use of heat shock, chemical and tissue specific promoter for the autoexcision of marker gene may vary in different plant systems and needs to be tested.

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