Marker free approach for developing abiotic stress tolerant transgenic *Brassica juncea* (Indian mustard)

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Abstract: - Use of selectable marker genes that confer resistance to transgenic plants on selection media is an extra burden on plant genome and also makes transgenic plants unacceptable to the consumer. The present study was taken to construct a novel vector for introducing, the *glyoxalase I* (*gly I*) gene under an inducible promoter to develop marker free salt tolerant *Brassica juncea.* The overexpression of the *gly I* gene has earlier been shown to impart salt and heavy metal stress tolerance in transgenic tobacco. The construct used in this investigation had the plant selectable marker gene *npt II* flanked by the *lox*P sites, together with the *cre* recombinase gene under the control of the heat-inducible (*hsp*) promoter. The *gly I* gene was cloned under a salt and drought inducible promoter (*rd29A*) so that it expressed only when the plant experienced stress. Using this novel construct, transgenic plants of *Brassica juncea* could be generated. The heat shock induction resulted in the excision of the intervening region between the two 34 bp *lox*P sites, which contained the *npt II* marker gene and the *hsp-cre* gene in the plants transformed with this vector. This strategy has been used to obtain marker free abiotic stress tolerant *Brassica juncea,* an economically useful oil yielding crop plant which could open the way for creating value added transgenic crops.

Key-Words: - *Brassica juncea,* glyoxalase I, Cre-*lox*P, *rd29A*, abiotic stress.

1. Introduction

Soil salinity is one of the most serious factors limiting productivity of agricultural crops worldwide. More than 60 million hectares of irrigated land (representing 25% of the total irrigated acreage in the world) has been affected by salt. To overcome this problem, a multidisciplinary approach is required. Genetically engineering the plants for abiotic stress tolerance is a viable option. The goal of genetic engineering strategies is to manipulate gene expression in ways that produce the desired phenotypic effects. In this investigation, an attempt has been made to introduce the *glyoxalase I* (*gly I*) gene driven by an inducible promoter for enhanced salinity tolerance.

The enzymes, 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 step reaction, these enzymes act coordinately to convert cytotoxic methylglyoxal and other 2-oxoaldehydes to their 2-hydroxyacids using glutathione as a cofactor [1]. The primary physiological function of the glyoxalase system appears to be the detoxification of methylglyoxal (MG), which is mainly synthesized as a byproduct of carbohydrate metabolism. Overexpression of the *gly I* gene in the model plant, tobacco has been shown to impart tolerance to salt, drought and heavy metal stress [2]. However, constitutive overexpression of the transgene may compete for the building blocks and machinery needed for RNA and protein synthesis under conditions without stress. Thus, it seems desirable to generate plants with transgene expression driven by a stress-inducible promoter, so that the specific mRNA and proteins are not produced unless under stress conditions. Yamaguchi-Shinozaki *et al* [3] showed that the *rd29A* and *rd29B* genes were induced under conditions of high temperature, high salt or treatment with exogenous abscisic acid (ABA). They used the *rd29A* promoter which has the drought-responsive element (DRE) and ABA-responsive element (ABRE). The 9bp DRE element is involved in the first rapid response of *rd29A* to conditions of dehydration or high salt. Since the selectable marker genes used in plant transformation experiments do not serve any purpose after the transfer of the desired gene, several approaches for the excision of the marker gene are being utilized. These include Flp/frt from *Saccharomyces* [4], R/RS from *Zygosaccharomyces* [5], Gin/gix from bacteriophage Mu [6] and Cre/*lox*P system from bacteriophage P1 [7] [8] [9] [10] [11] [12]. In this study , a simplified approach was used, wherein the *cre* gene was also cloned within the site in which *npt II* gene had been cloned so that by a single transformation both the *cre* gene and the *lox*P gene could be transferred to the

target species together with the gene of interest. Since, the ectopic expression of the *cre* gene could affect the plant growth, it was cloned under an inducible promoter [13].

Brassica juncea (Indian mustard) belongs to the family cruciferae. There are nearly 40 different varieties of this yellow flowering plant that botanists classify into the genus *Brassica* of which *Brassica juncea* is one of the major oilseed crops of India. The oil content varies between 35% and 45% and the protein content is between 20% and 24%. The seed residue is used as cattle feed and in fertilizers. It is a high biomass crop and also helpful in bioremediation of heavy metals in the polluted soils. A vast tract of agricultural land in India is presently non-arable due to excessive salinity and an effort to generate salt-tolerant *Brassica juncea* is a prime target for ensuring increased productivity.

In this investigation, *Brassica juncea* was transformed with the binary construct in such a way that it led to the generation of abiotic stress tolerant marker free transgenic plants.

2 Material and methods

2.1 Vector construction

The binary plasmid p*CAMBIA2301* was used as the cloning backbone. It contains the *CaMV35S* promoter-driven *neomycin phosphotransferase II* (*npt II*) gene, conferring resistance against kanamycin to the plants. The 34 bp *lox*P sequence was introduced both upstream of the *35S* promoter at the *Eco*RI site and between the *npt II* coding region and a *35S* terminator at *Xho*I site by ligating the *lox*P site-containing oligonucleotides in a two-step cloning procedure. The oligos were designed in such a manner that the restriction sites used for the cloning were preserved. The resulting sequences of the inserts with flanking vector nucleotides were as follows:
gagctcGAATTGATCTTCGTATAATGTATGCTA TACGAAGTTATgaattc-35S promoter (*Sac*I and *Eco*RI sites underlined); tcgagATAACTTCGTATA ATGTATGCTATACGAAGTTATGTCGAGtttctc-35S terminator (*Xho*I site underlined). The positive clones were confirmed by restriction digestion and sequencing. A 950 bp fragment of *rd29A* promoter (D13044) was amplified from *Arabidopsis thaliana* cv. Columbia genomic DNA with primers having *Xba*I and *Nco*I overhangs (5' GAGCTCTAGATGC AATTCAATCAAACTG 3'and 5' TGATCCATGG TCCAAAGATTTTTTCTTTCC 3'). The *gly I* gene (Y13239) from *Brassica juncea* was PCR amplified using the primers having *Nco*I and *Bst*EII overhangs (5' TTCTCCATGGCGTCGGAAGCGAAGGAATC 3' and5' TTTTGGTCACCGATAACAACTTATTT AACTCAACTC 3') from p*BS* vector containing the *gly I* cDNA sequence [2]. The *npt-lox* binary vector was digested with *Xba*I and *Bst*EII. This 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 *gly I* gene. The *hsp-cre* fragment with *Eco*RI and *Bam*HI overhangs was amplified from p*Crox* 18 vector [15] with the primers (5' GCCAGAATTCATCGGTTTGAAGAT GGCAAGTGTT 3'and 5' AATTGGATCCTAATCG CCATCTTCCAGCA 3'). The *CaMV35S* terminator was isolated from *dsProA* [16] by digestion with *Eco*RI and *Bam*HI. The above construct containing *nptII-lox+rd29A-glyI* was digested with *Eco*RI and dephosphorylated. A three fragment ligation was done with the above to get the final construct designated as p*hsp-cre-nptII-lox-rd29A-glyI*. The integrity and orientation of the double insert was confirmed by restriction analysis and later by sequencing (Fig. 1).

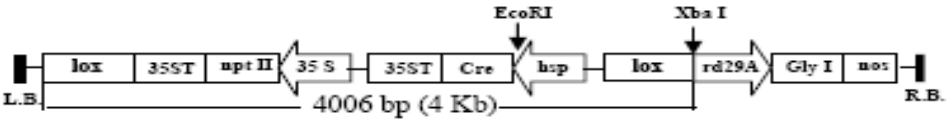

Fig. 1 Schematic representation of T-DNA portion of p*hsp-cre-nptII-lox-rd29A-glyI* vector used for the transformation of *Brassica juncea* cv. Varuna. The 4 kb fragment gets excised after the heat induction of *cre* recombinase gene.

2.2 Plant material and growth conditions

The vector (p*hsp-cre-nptII-lox-rd29A-glyI*) was introduced into *Agrobacterium tumefaciens* strain GV3101 by freeze thaw method. *Brassica juncea* cv. Varuna was transformed *via Agrobacterium* harbouring this vector. The seeds were germinated in culture tubes on MS semi-solid medium in dark after which they were transferred to 16:8h light:dark photoperiod. Hypocotyl explants (approx. 0.8-1.0 cm length) were prepared from five day old seedlings and inoculated on MS B_1N_1 semi-solid medium in petriplates containing BAP and NAA (1.0 mg/l each) with 2% glucose supplemented with kanamycin (40 mg/l). Filter sterilized $AgNO_3$ (3.5 mg/l) and gentamycin (200 mg/l) were also added. Well developed shoots were transferred to semi-solid MS I_2 root induction media (MS medium supplemented with 2 mg/l IBA) with kanamycin (40 mg/l). The putative transgenic plants were hardened in agropeat and transferred to green house for seed set (Fig. 2).

2.3 Heat induction treatment of transgenic *Brassica juncea* plants

The T1 seeds from PCR positive T0 plants and untransformed control plants were germinated on MS medium with and without kanamycin (40 mg/l) respectively. Two months old putative transgenic plants were given a heat shock in two phases. First, the T1 transgenics were treated at 37^0C for 24 h, 80% humidity level and allowed to recover at 23^0C in the culture tubes. After recovery, these plants were transferred to autoclaved agropeat and second heat shock was given at 40^0C for 24 h, 80% humidity level and left to recover again at 23^0C. Plants in small pots withstanding the heat shock were transferred to green house. After one month the transgenic plants were transferred to autoclaved soil and agropeat mixture for seed set.

2.4 DNA isolation, PCR and Southern hybridization

DNA was isolated from the leaves of transgenic as well as untransformed control plants by CTAB method [17]. About 10-15µg of genomic DNA was digested with *Xba*I overnight and blotted onto the nylon membrane as per the protocol of Sambrook *et.al* [14]. Genomic DNA (100 ng) was used as the template for PCR using the *npt II* and the *gly I* gene specific primers. The 680 bp region of the *gly I* gene was amplified using the primers: (5' GGGGTACCATGGCGTCGGAAGCGAAGG 3' and 5' TGCTCTAGCGCTCTCAAGCTGCGTTTC CGGCTG 3') and the 700 bp *npt II* gene coding region was amplified using the primers (5'GGAGCGGCGATACCGTAAAGC 3' and 5' GAGGCTATTCGGCTATGACTG 3'). PCR conditions were: initial denaturation at 94^0C for 30s, 55^0C (for *gly I*) or 58^0C (for *npt II*) for 30s and 72^0C (for *gly I*) for 1 min, 72^0C (for *npt II*) for 30s. The number of cycles was 35. For Southern analysis, the genomic DNA was digested with *Xba*I and probed with radiolabeled *npt II* or *gly I* cDNA. The DNA gel blots were probed with a ^{32}P- dCTP- labeled *gly I* cDNA.

3 Results

3.1 Transformation and selection of *Brassica juncea* transformants

The putative transgenic plants regenerated on selection medium were screened for the presence of the *gly I* and the *npt II* gene by PCR using gene specific primers. Approximately, 70% (7 out of 10 seeds) of the T1 transgenic *Brassica juncea* plants gave bands corresponding to ~680 bp for the *gly I* and ~700 bp for the *npt II* gene (Fig. 3 A & B). No

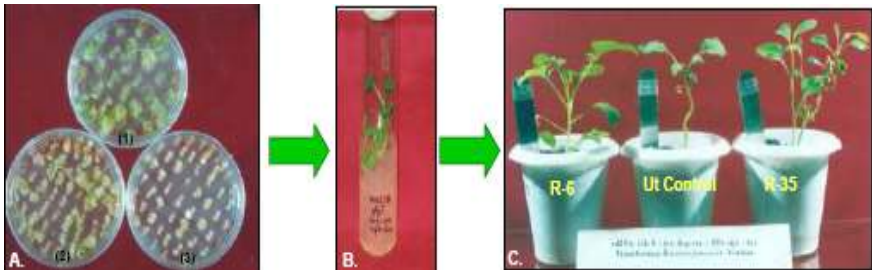

Fig. 2 Different stages of *Brassica juncea* transformation. A. Hypocotyl explants on MS B_1N_1 semisolid medium containing kanamycin; B. Putative transgenic shoots in root induction medium; and C. Putative transgenic plants in agropeat for hardening before transferring to the green house.

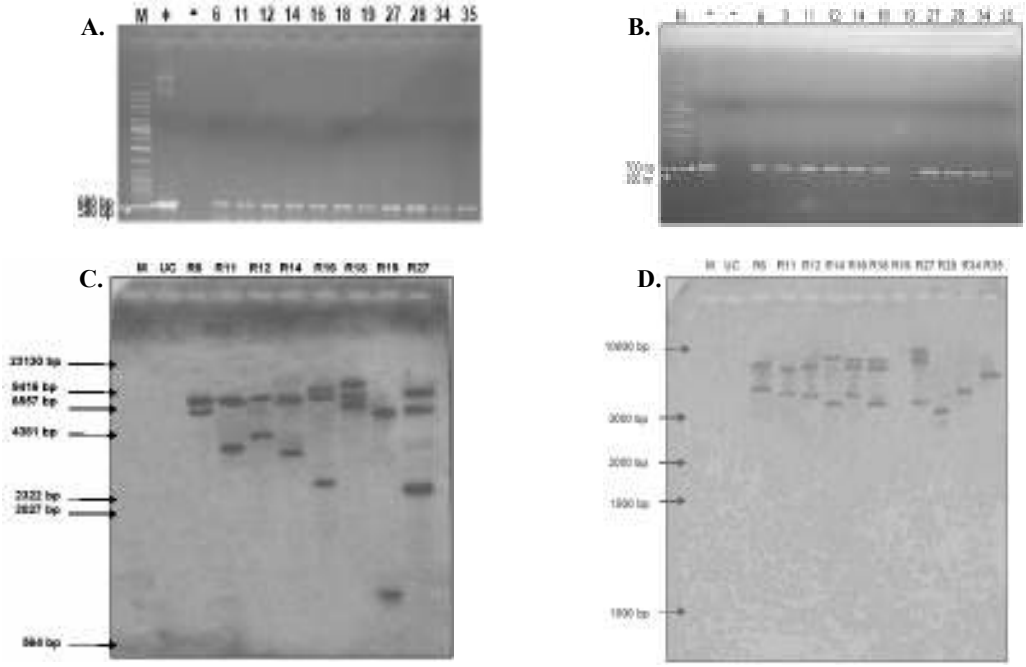

Fig. 3 A & B. PCR analysis for the *gly I* and the *npt II* genes respectively in T0 transgenic *Brassica juncea* transformed with p*hsp-cre-nptII-lox-rd29A-glyI* construct prior to heat shock treatment. M denotes DNA ladder; (+) is the plasmid DNA used as positive control; (-) is the negative control. C & D. Southern blot of the same transgenic *Brassica juncea* plants probed with *gly I* and *npt II* cDNA respectively.

corresponding bands were obtained in the untransformed control plants. The integration of these genes (the *gly I* and the *npt II*) in the transgenic plants was also confirmed by Southern blot analysis using radiolabeled *gly I* and *npt II* cDNA probes, respectively (Fig. 3 C & D). These transgenic plants grew well, flowered and set seed. The T1 seeds from these plants were germinated for performing MG, NaCl and $ZnCl_2$ tolerance tests as well as for marker excision studies.

3.2 Marker excision

T1 representatives of each of the PCR positive lines were used for heat shock induction studies (see Materials and methods). The plants subjected to heat shock flowered and set seed. Two transgenic lines showed excision of the 4 Kb fragment (see Fig. 1) after the heat shock at 37^0C for 24 h followed by transfer to 40^0C for 24 h. The PCR of these transgenic plants as well as the untransformed controls was carried out using the *npt II* and the *gly I* gene specific primers respectively. All the transgenic plants showed the band corresponding to the *gly I* gene. However, in two of the transgenic lines there was no amplification with the *npt II* primers (Fig. 4). These results showed that heat shock treatment caused excision of the marker gene *npt II,* but retained the gene of interest, the *gly I* gene in these two transgenic lines.

3.3 Leaf Disc Assay for tolerance against methylglyoxal, heavy metal and salinity stress

Healthy and fully expanded leaves from T1 generation of untransformed control and transgenic plants (30 days old) were briefly washed in deionized water. Leaf discs of 1 cm diameter were punched out and floated in a 5 and 10 mM solution (10 ml) of MG for 72 h; $ZnCl_2$ (20mM for 5 d) and NaCl (400, 600 and 800 mM for 5 d) or sterile distilled water for corresponding number of days (which served as the experimental control). The chlorophyll content was measured as described by Arnon [18]. The transgenic plants tolerated 10mM of MG (upto 3 d), 20 mM $ZnCl_2$ and 800 mM NaCl (as observed after 5 d respectively) while the untransformed controls bleached earlier (Fig. 5).

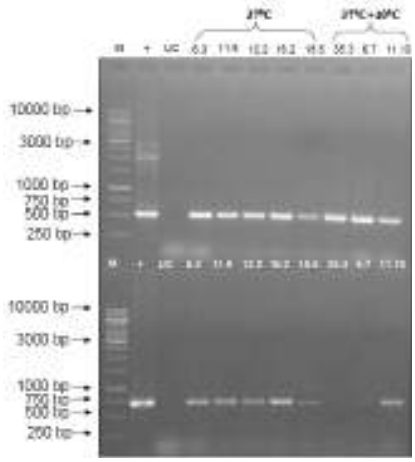

Fig. 4 PCR of T1 transgenic *Brassica juncea* plants with *gly I* (upper panel) and *npt II* (lower panel) gene specific primers after the heat shock treatment.

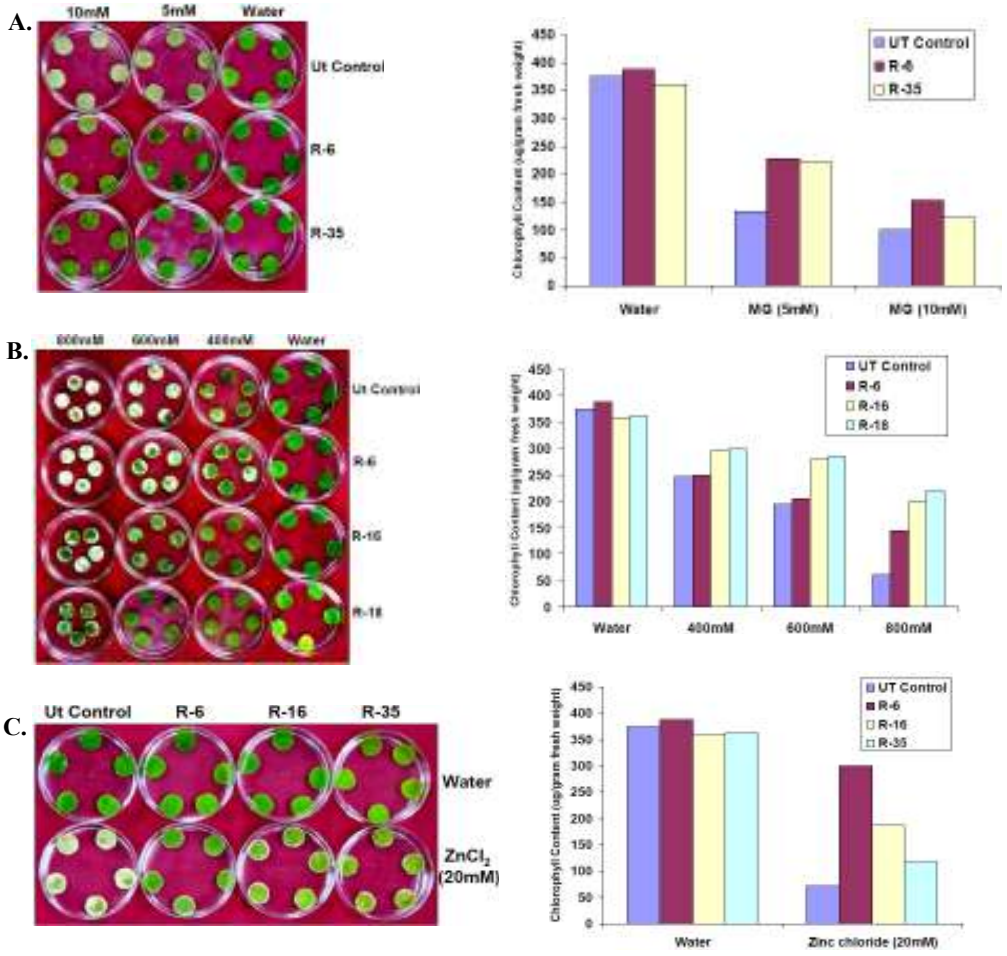

Fig. 5 Leaf disc tolerance test (left panel) and the corresponding chlorophyll content (right panel) of T1 transgenic *Brassica juncea* plants A. methylglyoxal treatment; B. NaCl treatment and C. $ZnCl_2$ treatment.

3.4 Comparison of salt stress tolerance in transgenic vs. the untransformed control plants in the green house

The transgenic plants were grown in the green house and irrigated with 200mM NaCl until the flowering stage. The transgenic plants showed better growth as compared to the untransformed control plants (Fig. 6). The transgenic plants were healthy and normal in morphology, flowered and set seed on saline soil whereas the control plants had stunted growth.

Fig. 6 T1 marker free transgenic *Brassica juncea* plants transformed with p*hsp-cre-nptII-lox-rd29A-glyI* vector growing under salinity stress (200mM NaCl) in the green house.

4 Conclusion

Modulation of the glyoxalase system has been shown to confer salt stress tolerance in transgenic tobacco plants [19] [2]. In contrast to the constitutive promoter used by other workers, the inducible *rd29A* promoter [20] which has two important *cis* elements-ABA responsive element (ABRE) and drought responsive elements (DRE) which mediate the stress responses was used in this investigation. The use of this novel vector construct led to the generation of antibiotic marker free salt tolerant *Brassica juncea* plants which would be more acceptable to the consumers. Since the successful excision of the *npt II* gene was obtained after the heat shock treatment of the transgenic lines, this strategy and the construct can be utilized for generating antibiotic marker free transgenic plants in a single step, thus hastening the recovery of the desired transgenic plants.

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