Transformation of *Vigna mungo* (blackgram) for abiotic stress tolerance using marker free approach

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Abstract

Salt stress is a major abiotic stress adversely affecting the yield of *Vigna mungo* (blackgram), an important pulse crop. In conjunction with cereals, it satisfies the dietary protein requirements of the predominantly vegetarian Indian population. Because of a narrow genetic base in pure-line selections and limited natural variation for desirable characteristics, a viable option for improvement of blackgram is through genetic engineering. The focus of our study has been on engineering salt-tolerance in blackgram by the over-expression of the Glyoxalase 1 (Gly 1) gene. Efficient regeneration systems have been established in *V. mungo* using different explants. Binary vectors were constructed wherein the Gly 1 gene was placed under the control of a constitutive (cestrum virus) and a salt-inducible promoter (rd 29A) respectively. The selectable marker gene (kanamycin resistance) in these vectors was flanked by lox sites so as to enable its excision following the recovery of transformed plants. Vectors containing the phosphomannose isomerase (PMI) gene were also used for transformation of *V. mungo*. Putative transformants showed tolerance to 600 mM NaCl and 10 mM methyl glyoxal, the substrate of glyoxalase 1 enzyme, compared to untransformed controls which did not survive on more than 200 mM NaCl and 5 mM methylglyoxal. They tested positive with the Gly 1, kanamycin and GUS primers. The transformants carrying the cre gene are being developed to cross with those containing the loxed kanamycin gene to obtain salt-tolerant marker free plants.

Media summary

Salt tolerant transgenics have been obtained in *Vigna mungo* using novel constructs which can be further used to recover marker-free plants.

Key Words

Legume, transgenics, POSITECH system, cre-lox.

Introduction

Vigna mungo (blackgram) is an important grain legume grown in the tropical and subtropical regions of the Indian subcontinent, and is adversely affected by saline soils. Due to lack of natural variation for abiotic stress tolerance, which limits breeding strategies, a genetic engineering approach has been used in *Vigna mungo*. Grain legumes are comparatively recalcitrant to regeneration and transformation. The present study highlights the establishment of reproducible and efficient regeneration systems and their amenability to genetic transformation in *V. mungo*. The Glyoxalase 1 (Gly 1) gene has been chosen for introduction in *V. mungo* as the overexpression of this gene in the model plant tobacco has been earlier shown to impart salt, drought and heavy metal tolerance (Veena *et al*. 1999). While it is important to insert an agronomically desirable gene to overcome the limitiations imposed by traditional plant breeding, emphasis should also be given to strategies that minimise the presence of recombinant DNA sequences to address public concern on GMOs and to achieve controlled expression of the transgene. The research work has been directed to use the POSITECH system (Todd and Tague 2001) as well as the cre-lox system to subsequently obtain marker-free, salt stress tolerant, transgenics of *V. mungo.*

Methods

Explant preparation for regeneration

Mature seeds of *V. mungo* were sterilized and imbibed in distilled water for 16-18 h at 90 rpm in a rotary shaker. The seed coat was removed and the cotyledons separated from the embryonic axis. The cotyledonary node was used as the explant after cutting off the shoot-tip and the root-tip from the embryonic axis. The resulting shoot-tip and de-embryonated cotyledons were also used as explants. The seeds were bisected into two equal halves and each embryonated cotyledon was used as an explant.

Construction of binary vectors

Two lox sites were inserted on both sides of the kanamycin resistance gene in the binary vector pCambia 2301 to obtain the vector pnpt-lox. The Glyoxalase 1 (Gly 1) gene driven by a novel constitutive cestrum viral promoter (Stavolone *et al*. 2003) was cloned in npt-lox to generate the modified binary vector npt-lox + csm-Gly1. The npt-lox vector was also used for cloning the Gly1 gene under the control of a salt-inducible rd 29A promoter (Kasuga *et al*. 1999). This was referred to as pnpt-lox + rd 29A-Gly1. The basic vector pNOV 2819 (Syngenta, USA) containing the *man A* gene coding for the enzyme phosphomannose isomerase (pmi) was used for cloning the casette containing Glyoxalase 1 (Gly 1) gene driven by the cestrum viral promoter to obtain the binary vector POSITECH-Gly1.

Genetic transformation

Binary plasmids were introduced into *Agrobacterium tumefaciens* strain GV 3101 using the freeze-thaw method. *Agrobacterium* cells were grown for 18 h before using the cultures for cocultivation with the regenerable explants following which the explants were transferred to selection medium. For pnpt-lox + csm-Gly1, kanamycin (75 mg/l) was used as selection agent. Prior to using POSITECH-Gly1, for transformation, untransformed explants were first tested for their growth on mannose containing medium. The explants were transferred to fresh selection medium at biweekly intervals.

Physiological analysis of transformants

Tolerance tests were performed by exposing leaf discs isolated from control and transgenic plants to sodium chloride (200 mM, 400 mM, 600 mM and 800 mM NaCl) and methylglyoxal (5 mM, 10 mM and 15 mM) solutions for 24 h, 48 h and 72 h. The effect of these treatments was assessed by observing phenotypic changes and by estimating the chlorophyll content in the leaf discs.

Results

Establishment of regeneration systems

Efficient and reproducible regeneration systems were established from embryonated and de-embryonated cotyledons, cotyledonary nodes and shoot-tips. Continuous exposure to BAP (6-benzyl amino purine) was important for maximal regeneration from cotyledon and shoot-tip explants, while TDZ (thidiazuron) was optimal for shoot bud induction from de-embryonated cotyledons. Highest number of shoot buds (12 per explant) were obtained from de-embryonated cotyledons, while 3-5 shoot-buds per explant were induced from cotyledonary nodes. The shoots could be separated from mother explant, elongated, rooted and hardened on soil.

Figure 1. Regeneration from nodal explants obtained from 16 h imbibed seed.

Transformation

Of the different explant types coinfected with *A. tumefaciens*, nodal explants were best suited for the recovery of stable transformants. Putative transgenics could be recovered on 75 mg/l kanamycin . These plants flowered and set seed. GUS expression could be detected in leaves and anthers. Genomic DNA harvested from these plants displayed the presence of a 1.2 kb amplicon with GUS and a 680 bp amplicon with the Gly I primers when subjected to PCR analysis.

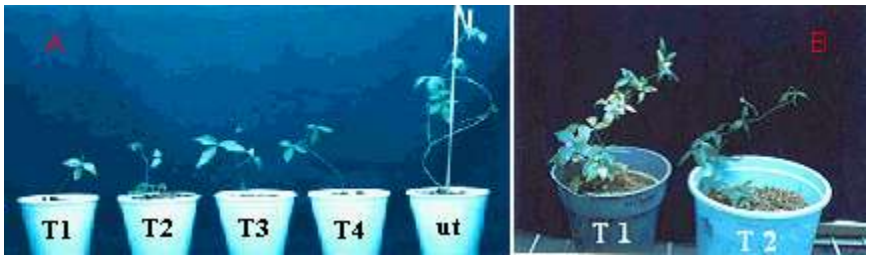

Figure 2. A. Untransformed control (ut) and transformed (T1-T4) plants in vermiculite B. Hardened transgenics in soil showing flowering.

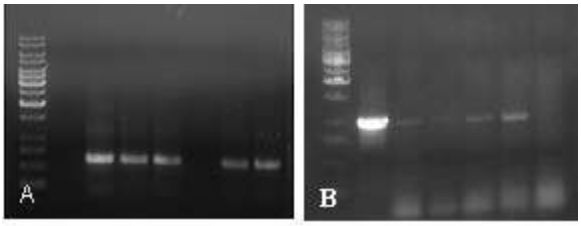

Figure 3. PCR analysis of transformed Vigna mungo plants A. Gly 1 primers (780 bp) B. GUS primers (1.2?kb).

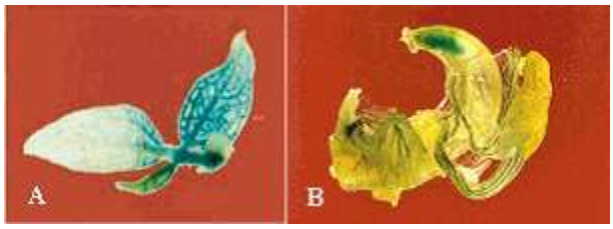

Figure 4. GUS expression in A. Leaves B. Anthers

Studies with mannose, a non-metabolizable carbohydrate source, as a positive selection agent indicated that the growth of only nodal explants could be suppressed on mannose-supplemented media and these could prove suitable for transformation using POSITECH-Gly 1. Explants transformed with pNOV 2819 grew on mannose-fortified medium while the untransformed controls perished.

Figure 5. A. Inhibition of untransformed nodal explants on mannose (M5, M7.5) compared to those on sucrose (S30). B. Explants transformed with pNOV 2819 (T) can grow on mannose-supplemented medium as compared to untransformed controls (UT).

Physiological analysis of transformants

Leaf discs from transgenic plants remained green on 600 mM NaCl and 10 mM methylglyoxal respectively while those from untransformed plants displayed marked bleaching. Noticeable bleaching was evident along the margins. The chlorophyll content (0.825 µg/g fresh weight) in transgenic leaf discs was 1.5 times more than that of the control leaf discs (0.555 µg/g fresh weight).

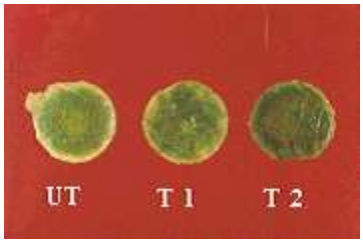

Figure 6. Leaf discs on 600 mM NaCl showing reduced senescence and bleaching of transformed (T1, T2) as compared to untransformed control (UT) .

Conclusions

Our studies led to the establishment of reproducible regenerable systems from different explants of *Vigna mungo.* Cotyledonary nodes proved to be the ideal explant for obtaining stable transgenics. The transformants obtained on kanamycin with the vector pnpt-lox + csm-Gly 1 were found to be PCR positive with GUS, npt II and Gly I primers. These transgenics were tolerant to NaCl and methylglyoxal induced stress and retained more chlorophyll as compared to the untransformed controls under short term stress conditions. The positive selection system has been successfully used in *V. mungo,* a legume. Studies on obtaining marker-free plants using the cre-lox system are in progress.

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