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Does *Agro*-injection to soybean pods transform embryos?

Muhammad Zia[1]*, Waheed Arshad[2], Yamin Bibi[3], Sobia Nisa[3], Muhammad Fayyaz Chaudhary[3]

[1]Department of Biotechnology, Quaid-i-Azam University, Islamabad 45320, Pakistan
[2]Department of Biochemistry, Quaid-i-Azam University, Islamabad, 45320, Pakistan
[3]Department of Microbiology, Quaid-i-Azam University, Islamabad, 45320, Pakistan

***Corresponding author: ziachaudhary@gmail.com**

Abstract

In planta and agroinfiltration technologies for plant transformation have gained attention in recent years. These technologies pass laborious tissue culture steps but bear low transformation efficiencies. Over-night grown *Agrobacterium tumefaciens* EHA105 harboring pROKIILFYGUSint plasmid was injected into soybean pods at three developmental stages. The seeds obtained were assessed for transformation through GUS histochemical analysis and PCR. Three plants of NARC-7 and two plants of NARC-4 (transformation efficiencies 14.2 and 6.45%, respectively) showed GUS activity in plant tissue when *Agrobacterium* was injected after 2-3 days of pod formation; i.e. stage I. Highest GUS expression (39.16 %) was observed on NARC-7 seeds when pods were treated at the late stage of development; i.e. stage III. To our knowledge, this is the first report that demonstrates transformation of developing embryo by pod agroinjection. Using this procedure, transformed seeds can directly be produced and can be further analyzed at progeny level.

Keywords: *Agrobacterium tumefaciens*, agroinjection, in planta, soybean, transformation.
Abbreviations GUS beta-glucuronidase; MS Murashige and Skoog; NARC National Agriculture Research Center.

Introduction

Plant genetic transformation permits the introduction of agronomically useful gene(s) into important crops and offers a significant tool in breeding programs by producing novel and genetically diverse plant materials. Generally three methods are responsible for transgenic plant production: indirect transformation mediated by *Agrobacterium*, direct systems using electroporation; and the biolistic. The natural mechanism of *Agrobacterium*-mediated gene transfer is widely used to genetically modify monocots and dicots (Hansen and Wright, 1999). During *Agrobacterium* mediated transformation, low transgenic plants produce due to a number of factors that include explant damage, biotic shock, antibiotic stress, inoculation time, long time culture that habituate callus/explant and many more (Mamidala and Nanna, 2009; Motamedi et al., 2011; Wagiran et al., 2010). Soybean has been proven extremely resistant to regeneration and transformation (Trick et al., 1997). Although soybean transformation has been attempted by the biolistic method (Finer and McMullen, 1991; Hazel et al., 1998; Santarem and Finer, 1999) and the *Agrobacterium* mediated methods (Clemente et al., 2000; Hinchee et al., 1988; Liu et al., 2004; Olhoft et al., 2001; Zhang et al., 1999; Zia et al., 2010a) over the past decades, the efficiency of transformation remained low. The methodologies used for soybean transformation are also associated with chimerism, and recovery of transgenic plants has not been very successful, even with soybean genotypes that are highly susceptible to *Agrobacterium* infection (Olhoft and Somers, 2001).

The development of in planta transformation system (Floral-dip method and Vacuum infiltration) radically accelerated research in basic plant molecular biology (Clough and Bent, 1998). By these methods meristem or other tissues that give rise gametes are targeted (Birch, 1997). Very few reports are available for transformation of leguminous crops through in planta methodologies. Trieu et al. (2000) obtained stable transgenics of *Medicago truncatula* (a model legume plant) by seedling infection and flower infiltration using *A. tumefaciens*. However, Li and coworkers were not successful in getting soybean transformants by pollen tube pathway. They stated that GUS activity expressed in some seeds was endogenous and DNA was inside the cell but not integrated into the chromosome (Li et al., 2002). Recently, Lu et al. (2009) reported 11% efficiency when styles were removed during in planta ovary transformation. Liu et al. (2009) dripped DNA onto the ovary wound after 6-8 hr of pollination. Maximum 3.2% transformation efficiency was observed. They also reported that ovary drip method produced more transformants than pollen tube pathway method. While, Li et al. (2010) inoculated *Agrobacterium rhizogenes* at vascular bundles of hypocotyls of four days germinated seedlings of soybean that produced chimeric plants. For soybean, improvement in transformation procedures is requisite. For this reason, the objective of this study was to develop tissue culture independent transformation procedure. In this paper a new technique, *Agrobacterium* injection (Agroinjection) to developing pod, was opted that passes laborious tissue culture steps.

Results

Soybean embryo and cotyledons were infected with *Agrobacterium tumefaciens* during various developmental

Table 1. Primer sequence of NPTII, GUS and LFY gene for PCR amplification

Gene	Primer sequence
NPTII	F-‘5-AAGATGGATTGCACGCAGGTTC-3’ R-‘5-GAAGAACTCGTCAAGAAGGCGA-3’
GUS	F-‘5-AACTGGACAAGGCACTAGCGG-3’ R-‘5-TGCGACCTGACCGTACTTGAA-3’
LFY	F-‘5-GTTGGTGAACGGTACGGTAT-3’ R-‘5-ACTAGAAACGCAAGTCGTCGTCG-3’

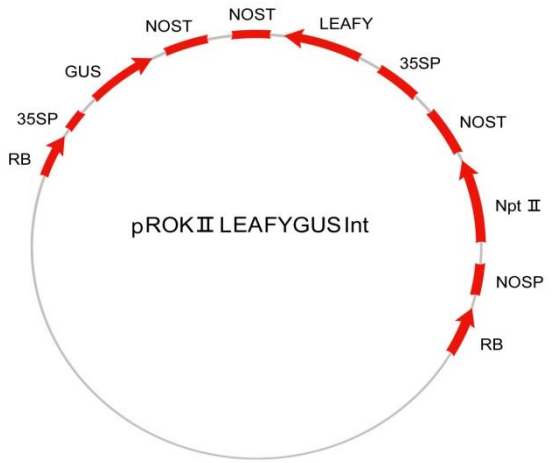


Fig 1. T-DNA region of transformation vector pROKIILFYGUSint. LB= Left Border, 35SP= 35 S Promoter, GUS= beta-Glucuronidase gene, NOST= 3-Nopaline synthase terminator, LFY= leafy gene, NOSP =3-Nopaline synthase Promoter, RB= Right Border

stages. Agroinjection to soybean pods was found efficient to produce transformed seeds. GUS histochemical analysis of seeds confirmed transformation of embryos and cotyledons. Plants germinated on selection medium also showed intense GUS expression on all parts .The summery of all experiments; agroinjection at different pod developmental stages is given in Fig 3.

Agroinjection at first stage of pod development

At first stage of pod development (stage I), total 383 pods (235 of NARC-4 and 148 of NARC-7) were infected with *Agrobacterium tumefaciens* strain EHA 105 and 105 viable seeds (62 of NARC-4 and 43 of NARC-7) were harvested. Out of 52 seeds (31 of NARC-4 and 21 of NARC-7) subjected to GUS assay. Seventeen seeds (11 of NARC-4 and 6 of NARC-7) showed GUS expression on seed (Fig 3). This showed transformation efficiencies 35.48 and 28.75% for NARC-4 and NARC-7, respectively (Fig 4). Out of 53 seeds (31 of NARC-4 and 22 of NARC-7) inoculated on selection medium, only 23 plants (14 of NARC-4 and 9 of NARC-7) survived. Out of these, two plants of NARC-4 and three plants of NARC-7 showed GUS expression on leaf and stem parts of plants; resulting in 6.45 and 14.2% transformation efficiency, respectively. Other than these five plants that showed complete GUS expression, some survived plants also exhibited GUS coloration only in their vascular tissues (xylem and phloem) while other tissues were negative for GUS response.

Agroinjection at second stage of pod development

In second experiment, when *Agrobacterium* suspension culture was injected after 8-10 days of pod formation (Stage II), a total of 473 viable seeds (264 of NARC-4 and 209 of NARC-7) were harvested at the end (Fig. 3). Only 62 seeds, out of 236, (38 of NARC-4 (28.78%) and 24 of NARC-7 (23.08%) showed blue coloration on seeds (cotyledons and embryo) (Fig. 4). Out of 83 plants (46 of NARC-4 + 37 of NARC-7) survived on selection medium, one plant of NARC-4 and three plants of NARC-7 were positive for GUS expression analysis defining 0.75% and 2.8% transformation efficiency, respectively (Fig 3 & 4).

Agroinjection at third stage of pod development

When soybean pods were treated with *Agrobacterium* at stage-III (late stage of pod development when seeds were fully mature but still green), genotype NARC-4 and NARC-7 showed 28.44% and 39.16% transformation efficiencies, respectively on the basis of GUS expression on seeds. A total of 229 seeds (109 of NARC-4 and 120 of NARC-7) were inoculated on selection medium for germination. Out of 45 plants (28 of NARC-4 and 17 of NARC-7) germinated on selection medium only one plant of NARC-7 showed GUS response describing 0.84% transformation efficiency (Fig. 4). The statistical analysis of data revealed that effect of developmental stages was significant for GUS expression on seed while it was non significant for GUS on plant tissue (Table 2).

Table 2. Effect of pod developmental stage on GUS expression in seed and in plant tissue.

GUS on seed	Variety	Number of GUS positive seeds			
		Stage 1	Stage 2	Stage 3	Mean ψ
	NARC-4	3.67	12.67	10.33	8.89 a
	NARC-7	2	8	15.67	8.56 a
	Mean *	2.833 B	10.33 AB	13 A	
	Variety	Number of GUS postive plants			
		Stage 1	Stage 2	Stage 3	Mean ψ
GUS on plant tissue	NARC-4	0.667	0.333	0	0.333 x
	NARC-7	1	1	0.333	0.778 x
	Mean ψ	0.833 a	0.667 a	0.167 a	

* Means significantly differ at 5% probability level. Means with similar alphabet did not differ significantly using LSD test
ψ Means are non significant at 5% probability.

Fig 2. Injection of *Agrobacterium* to soybean pods: (a) Agroinjection to stage I of soybean (b) cross section of pod at stage I (c) agroinjection to stage II (d) cross section of pod at stage II (e) agroinjection at stage III (f) cross section of pod at stage III (i) Seed morphology of soybean after repining (viable and non-viable seeds)

Maximum GUS expression efficiency on seed was recorded when pods were treated at developmental stages III followed by stage I and then stage II. However, highest transformation efficiency (14.2%) was observed when pods of NARC-7 were treated at stage I (Fig 3).

PCR analysis and phenotype of transformed plants

The plants survived on selection medium and GUS assay positive were subjected to PCR for amplification of GUS, NPTII and LFY genes. All the plants showing GUS expression were positive for the presence of all three genes (Fig 6). The plants showing GUS expression only at petioles also showed positive PCR results when DNA was extracted from complete leaf. Phenotypically all the plants were retarded, shortened height and no further branches were observed. Soybean leaves were normal as trifoliate but less in number (Fig 7). Three soybean plants produced flowers at the margin (Solitary inflorescence), that also hindered further growth of plant and no lateral branches were observed. These flowers started turning black from the pedicel and fall down before blooms without producing any pod.

Discussion

Present agroinjection method showed successful results for transformation of soybean: a legume crops. Such *Agrobacterium* injection to pod method is not reported in the past. Existing methods include floral dip method (Bent, 2000; Trieu et al., 2000; Qing et al., 2000); and pollen tube pathway (Li et al., 2002; Hu and Wang, 1999). In these methods exact time of incubation is very important because after locule closure, the pollen tube pathway blocks that hinders pollen tube carrying T-DNA to reach at its destination (ovule) (Desfeux et al., 2000). While, agroinjection carried out in fruits were aimed to analyze transient gene expression and in such reports seed or embryo transformation was not observed (Orzaez at al., 2006; Ahmad and Mirza, 2006; Spolaore et al., 2001). Agroinjection to pods; at initial days (stage I); showed higher GUS expression on seeds (cotyledons and embryo) and plant tissues germinated on selection medium. At early or late heart shaped embryo, the cells are differentiated for tissues formation (for review Jenik et al., 2007). If transformation occurs in the cells that are designated for shoot meristem; the regenerated plants must be transformed. While at the lateral stages (stage-II and stage-III) when the differentiation has been completed; transformation to cells at embryo tip may result in transformed plant. Agroinjection at early stage of pod development (within one or two days of pod formation) damaged the developing embryo at high ratio, resulting in less seed formation. At stage-II infection (when seeds were 3-4mm in diameter) more seeds were obtained and high expression was observed at embryo as well as on cotyledons but percentage of transformed plants was low. At late stage of seed development (when seeds have been matured but still green; stage-III); GUS expression in plant tissue was least but on seed was highest. Higher GUS activity on seeds than plants might be due to intrinsic GUS like

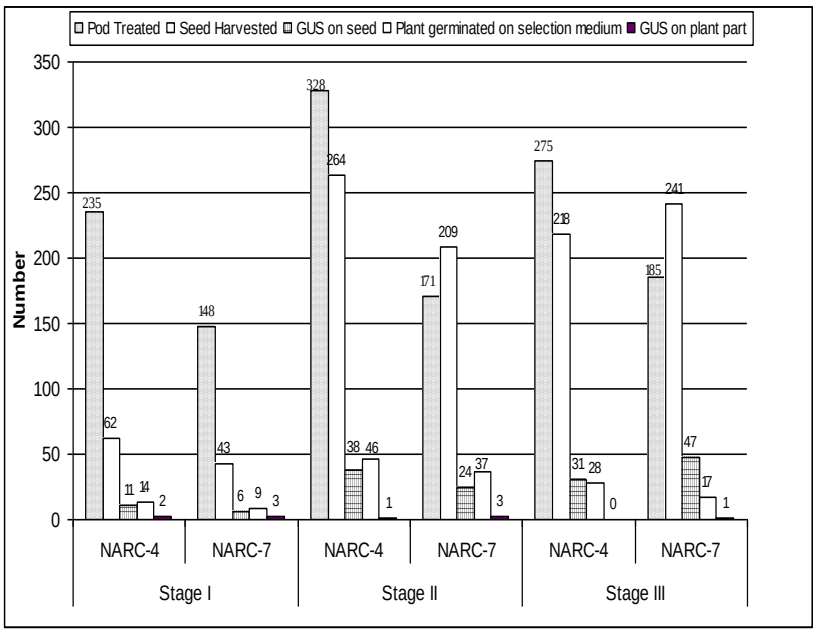


Fig 3. Summary *Agrobacterium* injection to two soybean cultivars (NARC-4 & NARC-7) at three different stages of pod development.

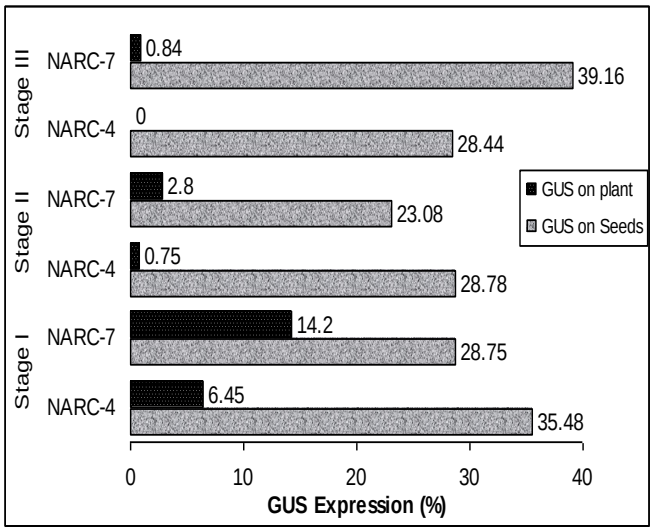


Fig 4. Transformation efficiency (%) of two soybean cultivars through GUS histochemical assay of seeds and plant parts

activity. Hu et al. (1990) reported GUS like intrinsic activity on mature and immature soybean seeds and also on other plant parts but failed to observe this false activity at flowering stage. Agroinjection to developing pod resulted transformation efficiency upto 14.2% while in recent reports maximum 3.2% transformation efficiency was calculated during in planta ovary transformation and 0.97% by pollen tube pathway transformation (Lu et al., 2009). While Liu et al. (2009) reported only 8.2% transformation efficiency on three soybean cultivars when DNA was dripped on ovary after style removal. The generated plants of soybean that showed GUS expression and presence of transformed gene; flowered nearly two week earlier due to insertion of extra copy of LFY gene but other plants did not produce flowers. It has been reported that LFY represses AGL24 which promote inflorescence fate rather then flower formation (Yu et al., 2004). While, Bermnier and Claire (2005) reported that genetic regulation of flower in shoot apical meristem defines floral transition as well as floral architecture. It also seemed that early flowering in soybean severly affected vegetative growth of plant and did not support the plants for pod formation. The results of plant phenotype (retarded growth and early flowering) are also in agreement of our results (Fig 7b) when soybean LFY transformants were produced through tissue culture method (unpublished data). Present methodology for *Agrobacterium* mediated transformation is easier than existing methods and produces viable transformed seeds because in other reports of agroinjection into fruits, no one reported transformation of embryo in seed while in floral dip method efficiency of transformation is low especially in leguminous crops. This method successfully produced transformed T0 plants without tissue culturing steps. Moreover, if transformation occurs only in cotyledons, these can be used as explant which minimizes the conditions standardization steps required for *Agrobacterium* infection and selection. These findings also conclude that direct contact of *Agrobacterium* with developing embryo by agroinjection can be an easy and efficient approach to get

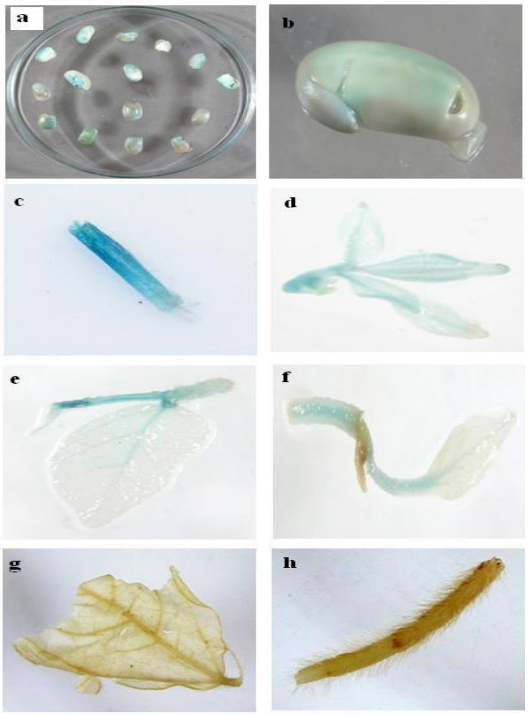


Fig 5. GUS staining on soybean after pod agroinjection (a) Soybean cotyledons (b) Complete soybean seed (c & d) stem explants (e & f) Soybean leaf explants (g & h) GUS expression only in vascular tissues (i & j) leaf and stem of untransformed explants (control).

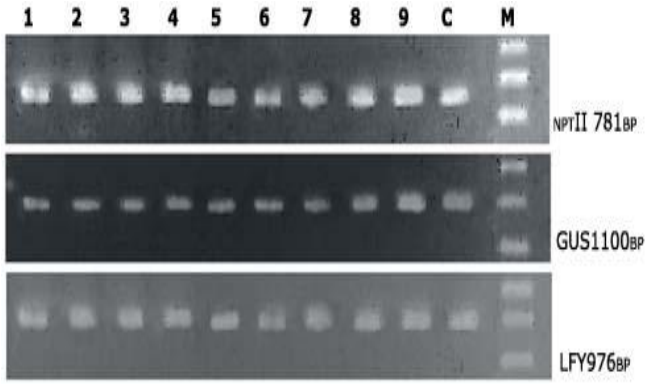


Fig 6. PCR analysis of Soybean plants to detect presence of NPTII, GUS and LFY genes: Lanes 1-9 = transformed soybean, C= plasmid (positive control) and M= 100bp marker.

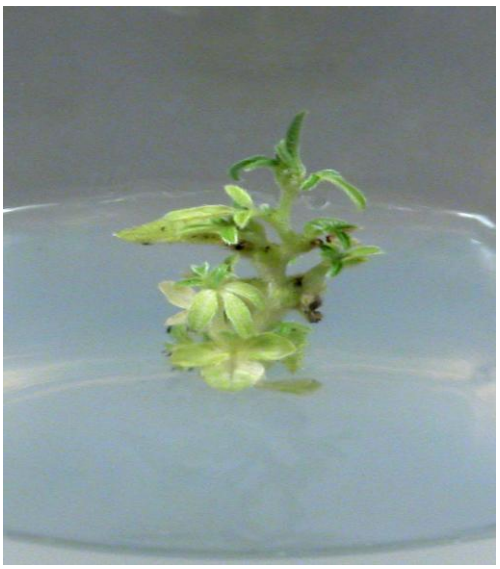
transformants but time of infection, concentration of *Agrobacterium*, use of surfactants and approaches to remove excess *Agrobacterium* after infection may be important factors to be studied.

Materials and Methods

DNA construct

The construct p*ROKIILFYGUSint* plasmid in *Agrobacterium tumefaciens* strain EHA 105 was used for transformation in this study. The T-DNA region contains LFY (Leafy) gene of *Arabidopsis* under the control of Cauliflower Mosaic Virus 35S promoter (CaMV35S) with GUS (beta-glucuronidase) gene as reporter and NPTII (neomycin phosphotransferase II) as selectable marker (Fig. 1). GUS gene contained an intron

(A)



(B)

Fig 7. Soybean LEAFY transformants (a) plant germinated from seed obtained after Agroinjection to pod (this study) (b) LEAFY transformants via tissue culture procedure using cotyledonary node method

to avoid expression in *Agrobacterium*. *Agrobacterium tumefaciens* strain was grown overnight in LB (Luria broth) medium containing 50 µg/ml kanamycin at 27°C and 200 rpm in incubator shaker. A volume of 100 µl was refreshed in 50 ml LB medium. The next day the culture was centrifuged at 4000 rpm (Eppendrof 5801R) and the pellet was re-suspended in MS liquid medium (Murashige and Skoog, 1962) of pH 5.7. The OD_{600} was adjusted at 1.0 by MS medium.

Plant material and transformation

Soybean cultivars NARC-4 and NARC-7 obtained from NARC (National Agriculture Research Center) were grown in plots containing leaf manure. Transformation procedure was performed at three stages of soybean pod development

with the help of 1-ml syringe with a 0.5 x 16 mm needle. In first experiment, *Agrobacterium* (500 µL) was injected in soybean pods within two days of pod formation; (stage-I) (Fig 2 a, b). In second experiment, inoculation of *Agrobacterium* (500 µL) to soybean pods was performed after 8-10 days of soybean pod formation (stage II), when soybean seeds were 3-4 mm in size (Fig 2 c, d). In third experiment, *Agrobacterium* was injected into fully developed soybean pods (stage III) before ripening (Fig 2 e, f). The pods injected with *Agrobacterium* were tagged and seeds were collected when ripened (Fig 2 i).

Selection of transformants

Half seeds from total viable seeds of soybean were soaked in distilled water for 6 hr and then surface sterilized with 0.1% mercuric chloride solution, rinsed thoroughly with distilled autoclaved water. The seeds were placed on ½ MS medium containing 100 mg/L Kanamycin for germination and the flasks were kept in growth room at 26°C, 10000 lux and 16 hr photoperiod. The plants survived on selection medium were subjected for GUS histochemcial analysis and PCR to confirm the presence of inserted fragment of T-DNA.

GUS histochemistry

After 21 days of seed germination, cotyledons, leaves and stem of soybean plants were used for histochemical GUS assay. The second half of total viable seeds (pre-soaked in distilled water for 12 hr) was also assayed for GUS activity. Histochemical staining of GUS activity was performed by incubating tissue sections and seeds in 1.0 mg/mL 5-bromo-4-chloro-3 indolyl -D-glucuronic acid, 0.1 M Na_2HPO_4 buffer (pH 7.0), 0.5 mM $K_3(Fe[CN]_6)$, 0.5 mM $K_4(Fe[CN]_6)$, and 10 mM EDTA as described by Jefferson et al. (1987).

PCR analysis of transformed plants

The plants survived on selection medium and showing GUS expression were subjected to polymerase chain reaction to confirm the presence of inserted fragment of plasmid. Leaf explants were used for extraction of genomic DNA by CTAB method as described by Zia et al. (2010b). PCR was performed for detection of NPTII, GUS and LFY genes. The primer sequences used for NPTII, GUS, and LFY genes are given in table 1. The PCR reaction mixture (25 µl) contained 1x PCR buffer, $MgCl_2$ (25 mM), dNTPs (0.5 mM), forward primer (10 µM), reverse primer (10 µM), Taq DNA polymerase (5 U), template DNA (100 ng total genomic DNA, or 10 pg plasmid DNA). The enzyme and primers were obtained from Fermentas (Maryland USA). The PCR reaction was conducted using an initial denaturation at 95°C for 4 min followed by 35 cycles of 94°C for 30 s, 53°C (for NPTII and GUS) or 54°C (for LFY) for 45 s, 72 °C for 45 s, and a final extension of 10 min at 72°C. The PCR products were analyzed on 1.0% agarose gels.

Statistical analysis

The experiments were conducted in triplicate using randomized complete block design (RCBD) and data obtained regarding the GUS on seed, germination on selection medium and GUS on plant tissue was statistically analyzed through ANOVA and LSD test using statistical software MSTATC v 2.0.

Acknowledgments

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