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RECOVERY OF amiRNA3-PARP1 TRANSGENIC MAIZE PLANTS USING A BINARY VECTOR HAVING THE BIOSAFE *PMI* GENE

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ABSTRACT

Positive plant selectable marker genes are commonly used in plant transformation because they not only enhance the frequency of generation transgenic tissues but are considered biosafe, unlike antibiotic or herbicide resistance genes. In this study, the binary vector pNOV2819-ubiamiRNA3PARP1, harbouring the phosphomannose isomerase (*pmi*) gene was developed and used in recovery of transgenic maize (*Zea mays* L.) plants containing the drought tolerance gene, amiRNA3-PARP1. The pre-amiRNA3-PARP1 and Tnos transgenes were sequentially PCR-cloned upstream the ubiquitine promoter in the Ubi/NC1300 plasmid. The pre-amiRNA3-PARP1 expression cassette was transferred into the *pmi* gene-containing pNOV2819 plasmid to produce the pNOV2819-ubiamiRNA3PARP1 vector. Transgenic IL3 and A188 plants containing pre-amiRNA3-PARP1 were generated through transformation with LBA4404 harbouring the pNOV2819-ubiamiRNA3PARP1 vector. The plants were confirmed transgenic by PCR. It is clear that the developed vectors are effective in recovery of amiRNA3-PARP1 transgenic tissues and plants containing the *pmi* gene, which has been shown to have no negative environmental or health effects.

Key Words: *Agrobacterium* transformation, amiRNA, PMI, *Zea mays*

RÉSUMÉ

Les marqueurs génétiques de sélection positive de plantes sont communément utilisés dans la transformation des plantes parce que, non seulement ils augmentent la fréquence de la génération des tissus transgéniques, mais aussi sont considérés comme biosains, à l'inverse des gènes de résistance aux antibiotiques et herbicides. Dans cette étude, le vecteur binaire pNOV2819-ubiamiRNA3PARP1 portant le gène isomérase phosphomannose (*pmi*) a été développé et utilisé dans le recouvrement transgénique des plantes du maïs (*Zea mays* L.) contenant le gène to tolérance à la sécheresse amiRNA3-PARP1. Les transgènes pre-amiRNA3-PARP1 et Tnos étaient séquentiellement clonés par PCR dans la partie supérieure du promoteur ubiquitine dans le plasmide Ubi/NC1300. L'expression de la cassette de la pré-amiRNA3-PARP1 était transférée dans le gène *pmi* contenant le plasmide pNOV2819 pour produire le vecteur pNOV2819-ubiamiRNA3PARP1. Les plants transgéniques IL3 et A188 contenant le pre-amiRNA3-PARP1 étaient générés à travers la transformation avec LBA4404 portant le vecteur pNOV2819-ubiamiRNA3PARP1. Les plants étaient confirmées transgéniques par PCR. Il est clair que les vecteurs développés sont efficaces dans le recouvrement des tissus transgéniques amiRNA3-PARP1 et les plants contenant le gène *pmi* qui ne présentent aucun effet négatif sur l'environnement et la santé.

Mots Clés: Transformation *Agrobacterium*, amiRNA, PMI, *Zea mays*

INTRODUCTION

A selectable marker gene (SMG) is an essential genetic component of a plant transformation vector. It is useful in identifying plant cells and tissues that take up foreign DNA during genetic transformation. Among the commonly used SMGs are those that code for antibiotic or herbicide resistance. These SMGs have been implicated in reducing the transformation efficiency for many crops. In addition, their use continues to raise environmental and food safety concerns (Miki and McHugh, 2004).

Today, the phosphomannose isomerase (*pmi*) gene is being used as a safer alternative to the antibiotic and herbicide resistance SMGs (Privalle *et al*., 1998; Jaiwal *et al*., 2002). The *pmi* gene was isolated from *E. coli* and has been used to recover transgenic plants at a higher frequency than antibiotic resistance genes (Joersbo *et al*., 1998; Dawson *et al*., 2001). It has been widely used in transformation of monocotyledonous plants, including wheat (Gadaleta *et al*., 2006; Dawson *et al*., 2001), rice (He *et al*., 2004), sorghum (Gao *et al*., 2005) and maize (Negrotto *et al*, 2000; Dawson *et al*., 2001).

The aim of this work was to prepare plant transformation vector containing the amiRNA transgene against maize *PARP1* gene and use it in maize transformation.

MATERIAL AND METHODS

DNA manipulation and cloning was achieved using protocols described by Sambrook *et al*. (1989). Restriction endonucleases and DNA polymerase were purchased exclusively from New England Bio-labs Inc., MA, USA. DNA ligases were procured from Invitrogen Corp. Carlsbad CA, USA. All oligonucleotides for PCR and sequencing were designed using the RealTime Design™ software (Biosearch Technologies), and synthesised in Bioneer Corporation (Korea) through Biosciences Eastern and Central Africa (BecA) Hub (ILRI, Nairobi, Kenya). DNA sequencing was done by the SEGOLI division of the BecA Hub. *Escherichia coli* and *A. tumefaciens* strain LBA4404 cells were made competent using the method of Tu *et al*. (2005) and Xu and Li (2008), respectively. Competent *E. Coli* and LBA4404 cells were transformed by the heat shock and freeze thaw (Raviraja and Sridhar, 2007) method, respectively.

PCR cloning of amiRNA1-PARP1 into Ubi/NC1300. The complete pre-amiRNA3-PARP1 region (414 bp) was amplified from pExamiRNAL2 (Fig. 1) vectors using the amiRNA forward primer (5' tatcacacccgggtccccaaacacacgctcg 3') having the *SmaI* restriction site and the amiRNA reverse primer (5' atacagagctctccccatggcgatgccttaa 3') having the *SacI* restriction site. All reactions were performed in volumes of 100 µl, comprising of ×1 PCR reaction buffer, 0.5 mM dNTPs, 2.5 mM $MgCl_2$ 0.25 M of each primer, 1 U Taq polymerase and 20 ng of plasmid DNA. Amplifications were performed on a Mastercycler vapoprotect (Eppendorf Hamburg, Germany) programmed with, 30 cycles of denaturation at 95 °C for 15 sec °C, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. An initial denaturation step at 95 °C for 5 min was performed. PCR products verified by agarose gel electrophoresis were purified using the Qiaquick PCR purification kit (Qiagen, Maryland, USA). PCR products were digested with *SmaI* and *SacI* and ligated into the *SmaI/SacI* site of Ubi/NC1300 plasmid (Fig. 1). The ligation mixture was transformed into competent *E. coli* cells and the positive recombinants harbouring the new Ubi/NC1300-amiRNA3 vector identified by *XbaI* digestion and sequencing.

Cloning of *Tnos* into Ubi/NC1300-amiRNA3 vectors. The full nos terminator sequence (288 bp) was amplified by PCR using the *Tnos* forward primer (5' tatcacagagctcgttcaaacatttggcaa 3') having *SacI* restriction site and Tnos reverse primer (5' atacaactagtgaattcccgatctagtaacatagat 3') having *SpeI* restriction site at their 5' ends. PRESQ101 (Fig. 1) plasmid was used as the template for the PCR reaction. The PCR amplicons were purified and ligated into the *SacI/SpeI* site of Ubi/NC1300-ubiamiRNA3. The ligation mixture was transformed into competent *E. coli* cells and the positive recombinants haboring the new Ubi/NC1300-amiRNA3Tnos vector identified by *HindIII/SpeI* digestion and sequencing.

Construction of pNOV2819-ubiamiRNA3 vector. A *HindIII/SpeI* fragment was removed

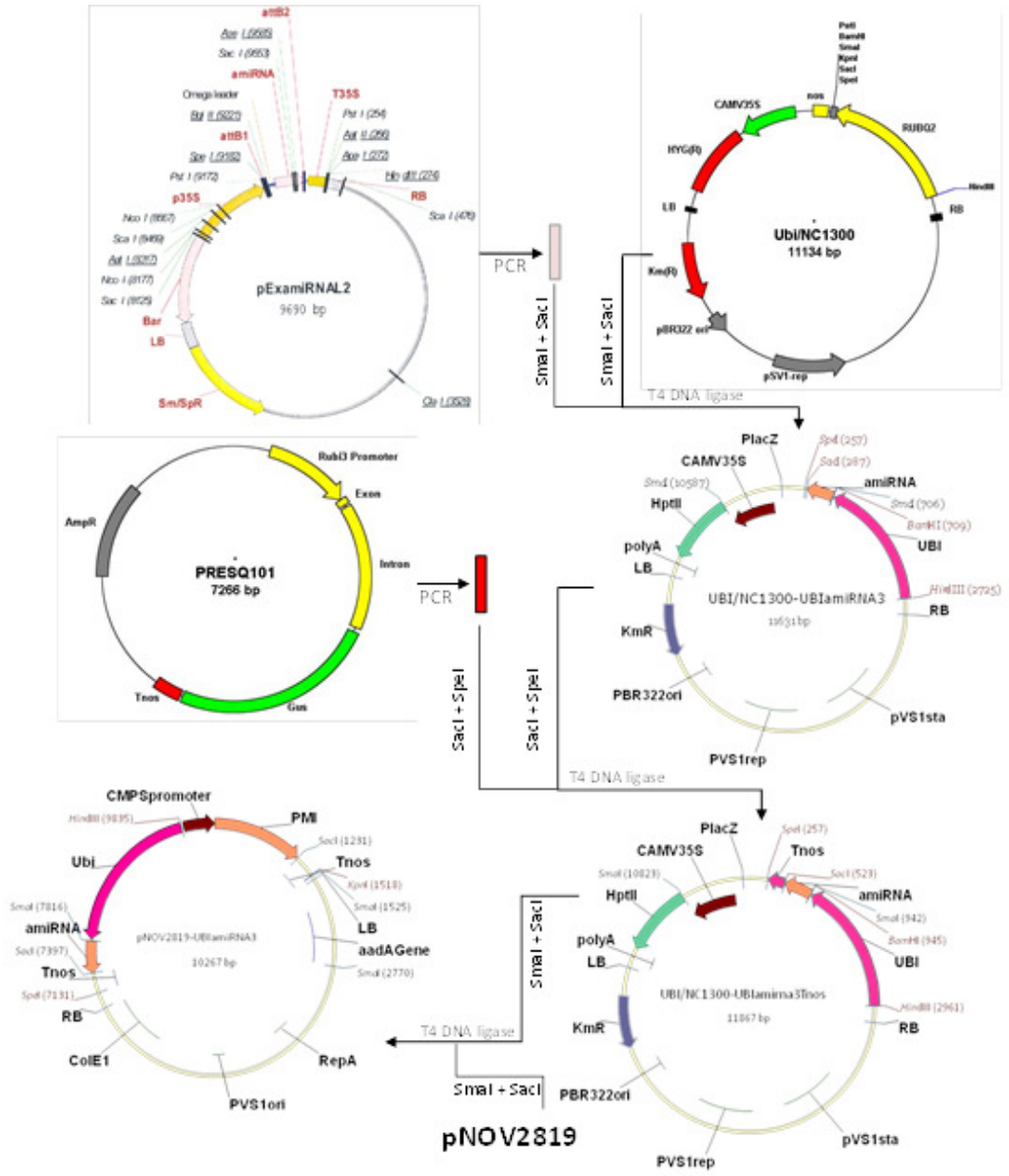

Figure 1. Scheme for construction of pNOV2819-ubamiRNA3 vector.

from Ubi/NC1300-ubiamiRNA3Tnos and ligated into the *HindIII/SpeI* site of pNOV2819 (SYGENTA Biotech Laboratories, North Carolina, USA). Ligation products were transformed into competent *E. coli* cells and positive transformants identified by digestion with *HindIII* and *SpeI* (Fig. 1).

Transformation of *A. tumefaciens* with pNOV2819-ubiamiRNA3 vector. The plant expression vector pNOV2819-ubiamiRNA3 was transformed into competent LBA4404 cells and the recombinant clones identified by PCR using primers specific to amiRNA3-PARP1 gene.

Preparation of PCR reactions and amplification conditions was done as described earlier.

Transformation of maize. LBA4404 harbouring the pNOV2819-ubiamiRNA3 vector was used to transform immature embryos of the inbred lines A188 and IL3. This was achieved using the cocultivation method of Negrotto *et al*. (2000). Transgenic callus tissues were selected on 0.5% mannose. The number of surviving callus was compared with the total number of infected cells to compute the transformation frequency (TF) for each genotype. Putative transgenic plants were regenerated from mannose-resistant tissues. To identify truly transformed plants, DNA was extracted from regenerants using the CTAB method (Saghai-Maroof *et al*., 1984) for PCR analysis. PCR was performed as described earlier using primers specific to the *PMI* gene (PMI-F 5'ctcgctgcatgaccttagtg 3' and PMI-R 5' ttgtaaacacgcgctaaacg 3').

RESULTS

Construction of Ubi/NC1300-ubiamiRNA3 vector. The full-length pre-amiRNA3PARP1 gene of 414 bp (Fig. 2A) was amplified successfully using amiRNA-PARP1-specific primers on pEXamiRNAL2 vector. The pre-amiRNA3-PARP1 gene was ligated onto Ubi/NC1300 vector and positive clones were identified by digestion with *XbaI* (Fig. 2B). *XbaI* released a 450 bp fragment from the recombinant plasmids. However, the band was absent in the control plasmid. The sequence of pre-amiRNA3PARP1 gene was validated by GeneBank database following sequencing of positive clones.

Construction of plant expression UBI/NC1300-ubiamiRNA3Tnos vector. Amplification products (726 bp) were observed only for the recombinant clones. However, PCR reactions for water and Ubi/NC1300-ubiamiRNA3 vector used as negative controls had no observable amplifications (Fig. 3A). Digestion of DNA with *HindIII* and *SpeI* resulted in release of a 2554 bp band from the recombinant clones (Fig. 3B, lane 2 and 3). However, this fragment was absent from the control vector (Ubi/NC1300-ubiamiRNA3), which lacks a cloned *Tnos* gene. Instead, the control vector released a band which was slightly smaller than the one released by the recombinant clones (Fig. 3B, lane 1). One positive clone, whose sequence of the cloned *Tnos* was validated by

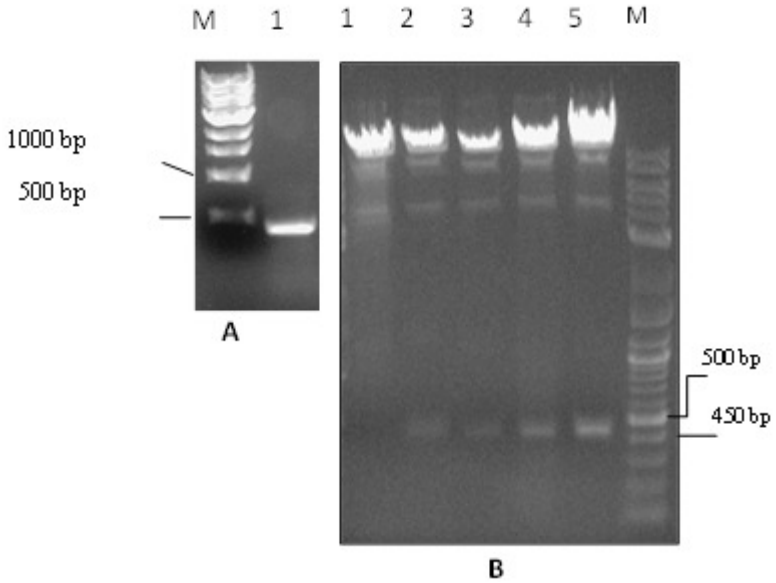

Figure 2. PCR amplification of pre-amiRNA-PARP1 and digestion of Ubi/NC1300-ubiamiRNA3. A: M: 1 Kb DNA ladder; A: 1 amplification of pre-amiRNA-PARP1; B: M: 1 Kb DNA ladder extra plus; B: 1: Digestion of control plasmid (Ubi/NC1300) with*XbaI*; B: 2 – 5: Digestion of Ubi/NC1300-ubiamiRNA3 with *XbaI*.

GeneBank database, was selected for use in development of the final vector, pNOV2819-ubiamiRNA3.

Construction of plant expression pNOV2819-ubiamiRNA3 vector. Results of PCR performed on DNA extracted from six colonies revealed the presence of the expected band (414 bp) in two colonies (Fig. 4A). Digestion of DNA from the PCR positive recombinant clones using *HindIII* and *SpeI* enzymes resulted in the release of the expected 2554 bp fragment from the recombinant plasmids (Fig. 4B, lane 2 and 3)and the control plasmid (Fig. 4B, lane 1).

Identification of recombinant *A. tumefaciens* LBA4404 cells that took up the pNOV2819-ubiamiRNA3 were identified by the presence of a 414 bp band in their PCR profile (Fig. 5).

Identification of transgenic maize plants. Transgenic tissues were recovered at different frequencies. The temperate line A188 had an

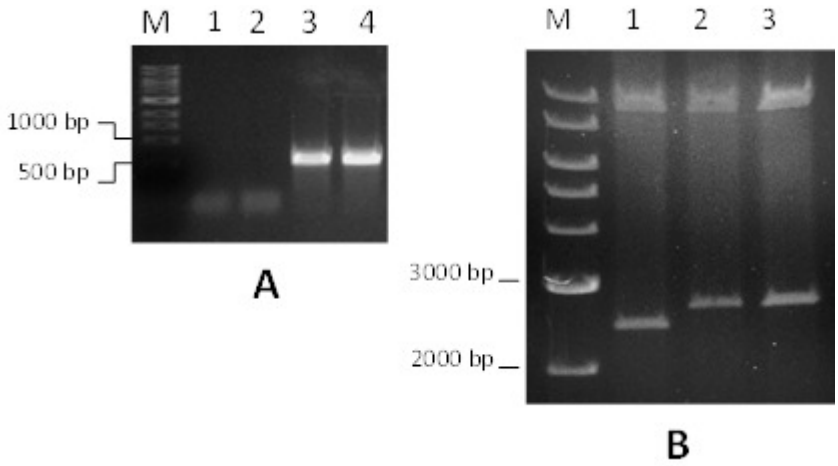

Figure 3. PCR amplification of Tnos and digestion of Ubi/NC1300-ubiamiRNA3Tnos. A: M:1 Kb DNA ladder; A: 1 amplification of pre-amiRNA-PARP1; B: M:1 Kb DNA ladder; B: 1: Digestion of control plasmid (Ubi/NC1300-ubiamiRNA3) with *HindIII* and *SpeI* restriction enzymes; B: 2 – 3: Digestion of Ubi/NC1300-ubiamiRNA3Tnos with *HindIII* and *SpeI* restriction enzymes.

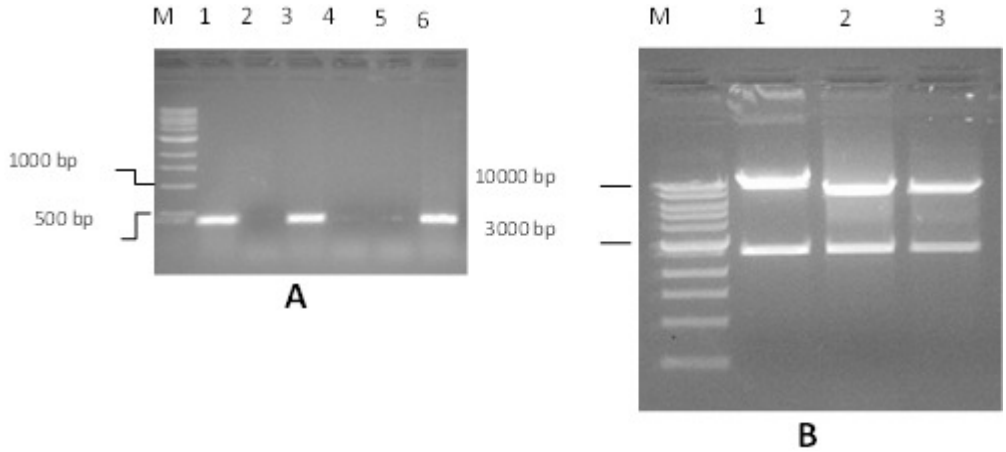

Figure 4. PCR amplification of pre-amiRNA-PARP1and digestion of pNOV2819-ubiamiRNA3. A: M: 1 Kb DNA ladder; A: 1 amplification of pre-amiRNA-PARP1 on 6 selected colonies; B: M: 1 Kb DNA ladder; B: 1: Digestion of control plasmid (Ubi/NC1300-ubiamiRNA3Tnos) with *HindIII* and *SpeI* restriction enzymes; B: 2 – 3: Digestion of pNOV2819-ubiamiRNA3with *HindIII* and *SpeI* restriction enzymes.

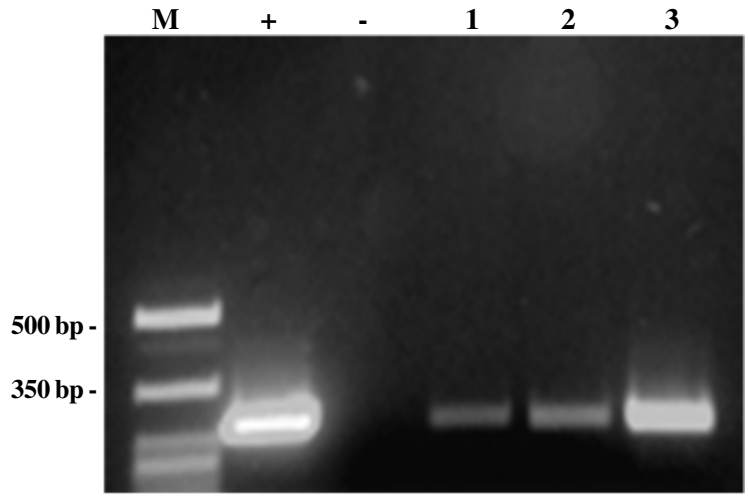

Figure 5. Amplification of the pre-amiRNA-PARP1 gene on plasmid DNA from different *A. tumefaciens* transformants. M: Low molecular weight DNA ladder; +: pNOV2819-ubiamiRNA3 used as positive control; -: Nontemplate control; 1-3: plasmid DNA from three selected *A. tumefaciens* transformants.

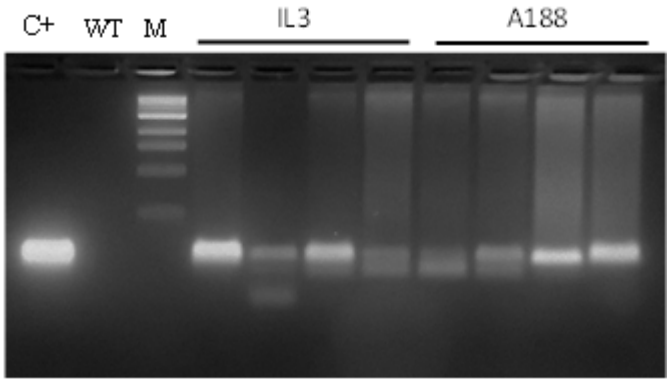

Figure 6. Identification of transgenic PMI plants C+: Positive control (pNOV2819-ubiamiRNA3 plasmid DNA); WT: Negative control (DNA from a non-transgenic plant) M: 1 KB DNA ladder. IL3: DNA from four putative transgenic IL3 plants; A188: DNA from four putative transgenic A188 plants.

average TF of about 3%, while the tropical genotype IL3 had a TF of 4.6%. The IL3 plant number 1 and 3 and the A188 plant number 3 and 4 were identified as transgenic due to the presence of an intense 200 bp amplicon in their PCR profile (Fig. 6).The four transgenic plants were selected for further analyses.

DISCUSSION

In this study, an amiRNA transgene targeting the maize *PARP1* gene was subcloned into pNOV2819 to produce the new binary vector pNOV2819-ubiamiRNA3PARP1. Using *A. tumefaciens* strain LBA4404, the new vector enabled integration of the *pmi* gene into the cells of maize genotypes A188 and IL3, subsequently enabling identification of transgenic tissues on mannose. The use of the *pmi* gene in plant transformation has enabled production of transgenic plants in different species including maize (Negrotto *et al.*, 2000), rice (Lucca *et al.*, 2001) and wheat (Wright *et al.*, 2001). The PMI protein has been found to pose no negative effects on humans or the environment.

Using *pmi*, Negrotto *et al.* (2000) recovered transgenic A188 maize tissues at frequencies of over 30%. Our comparatively lower

transformation frequency (3%) may be attributable to use of PNOV2819 as the vector backbone as opposed to the pNOV117 used in that study. In pNOV2819, the *pmi* gene is under the CMPS promoter which is weaker than the ubiquitine promoter driving the *pmi* gene on pNOV117 (Negrotto *et al.,* 2000). The stronger promoter may enable recovery of transgenic tissues at a higher frequency than the weaker one. In our study, transgenic tissues of IL3 were recovered at a rate of 4.6 %. IL3 has also been transformed using the negative selection gene *bar* (Rasha *et al*., 2013). However, presence of selectable marker genes in the final transgenic product continues to raise biosafety and environmental safety concerns (Jaiwal *et al.,* 2002).

Silencing of the *PARP1* gene is one of the recently developed genetic engineering strategies to enhance drought tolerance in plants. This approach has been shown to be very effective in model plants *A. thaliana* and *B. napus* (De Block *et al*., 2004; Schulz *et al*., 2012; Vanderauwera *et al*., 2007) and, therefore, hold great promise for agronomically important plants such as maize. In this study, we generated plants that had integrated amiRNA3 against maize PARP1 gene in their genome. Analysis of these plants for stable integration and expression of the *PMI* and amiRNA3-PARP1 transgenes is ongoing at the plant transformation laboratory (PTL) at Kenyatta University.

CONCLUSION

This study has generated plant transformation vector bearing an amiRNA targeting the maize PARP1 gene. These vectors are can be used to transform any maize genotype. For the first time, we have demonstrated using the *pmi* gene as the selectable marker gene, that the tropical maize inbred line IL3 is transformable with the amiRNA-PARP1 transgene. This study now paves way for introduction of different amiRNA-PARP1 transgenes into the pNOV2819 backbone for transformation of different tropical maize genotypes.

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