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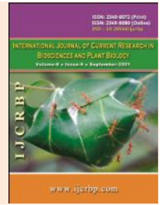

Original Research Article

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Inheritance of *NmDef02* synthetic defensin gene and *hpt* selectable marker in four successive generations of transgenic rice (*Oryza sativa*, cv. J-104)

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Article Info

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Abstract

The evaluation of the inheritance and stability of the transgenes is essential for the application of transgenic plants in agriculture. In this work, we studied the inheritance of two transgenes in T1, T2, T3 and T4 rice progenies. Transgenic rice plants (cv. J-104) was obtained by biolistic method using a synthetic defensin gene (*NmDef02*) and *hpt* as selectable marker gene, co-transformed in a 4:1 proportion, respectively. Regenerated plants were acclimated under natural conditions. The study started from the primary transformants that fulfilled the agronomic characters reported by the experts for the J-104 rice cultivar in the maturation stage. The integration and relative expression of *NmDef02* in T1 plants was verified by Southern blot and qRT-PCR, respectively. The inheritance of transgenes over four generations was analyzed by PCR. The following transgene combinations were identified: *NmDef02*(+)*hpt*(+), *NmDef02*(+)*hpt*(-) and *NmDef02*(-)*hpt*(-). The most advantageous combination was *NmDef02*(+)*hpt*(-), which corresponded to the marker-free plants harboring the gene of interest. The inheritance of *NmDef02* was confirmed in T1 and T2 progenies, but in some T3 and T4 lines the loss of this gene was verified. This inheritance analysis provided information concerning the transgenic population, but the mechanisms that destabilize the inheritance of the gene of interest will be the goal of future research.

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Introduction

The integration of a transgene into the plant genome is a random and complex event depending on the transgene itself and the host genome, and its expression may be stable or variable regardless of the method of gene transfer (Ahuja and Fladung, 2014). Determining the expression and inheritance of the transgenes is of paramount importance for the introduction of genetically modified plants in agriculture, since the main role of plant biotechnology is to improve the crops and, simultaneously, reduce the possible risk threats (Singh et al., 2019).

The application of genetic transformation technology to the plant breeding programs is sustainable if transgenes are stable and heritable over many generations. Both Mendelian and non-Mendelian transgene inheritance has been described through several generations of crop plants (Ahuja and Fladung, 2014). Once transgenes are

integrated into the host plant cells, events can occur that lead to opposite results: the transference of transgenes to further progenies through sexual generations while retaining the expression stability, or their loss, inactivation or silencing (Singh et al., 2019). Transgene integration sites exhibit different levels of structural complexity in the host DNA, and the primary transformants revealing consistent transgene expression may not give rise to progeny with the same characteristics (Betts et al., 2019).

From the 90's to date, several genetic-engineering-based strategies have been developed to generate transgenic rice with improved agronomic traits. More recent studies have been conducted to describe the inheritance and segregation patterns of transgenes, with the fundamental objective of detecting marker-free lines as well as evaluating the expression and stability of the gene of interest across the progenies (Xu et al., 2017; Feng et al., 2017; Xu et al., 2020; Li et al., 2021). The co-transformation method has been one of the most applied to obtain marker-free lines, using two distinct plasmid vectors: one containing the gene of interest and the other the selection marker gene that are inserted at two different loci in the genome. The selection marker could be eliminated by progeny segregation (Oliva et al., 2014). For the large-scale screening of transgenic rice progeny, there have been used highly suitable systems as the double fluorescent protein-expressing Ac/Ds vector applied by Li et al. (2021). These strategies have improved the reliability of the selection of transgenic segregating populations and reducing the rate of false marker-free transgenic plants.

The present work was carried out for evaluating the inheritance of the synthetic defensin gene *NmDef02* (Portieles et al., 2010) and the hygromycin-phosphotransferase (*hpt*) selectable marker gene in four successive generations of transgenic rice (cv. J-104), obtained by biolistic co-transformation of embryogenic calli. As a novel detail, the study started from the primary transformants that fulfilled the descriptive characters established for the plants of the traditional cultivar in the maturation stage.

Materials and methods

Plasmid construction

The plasmid pA9NmDef02 contained the gene of interest, which was a synthetic version of the defensin gene *NmDef02* (Portieles et al., 2010), isolated from *Nicotiana megalosiphon*. This gene was placed under the control of the A9 constitutive promoter, based on CaMV35S plus a quadruplicated *Agrobacterium* OCS enhancer element and the first exon/intron/exon of actin 1 gene from rice. The original sequences that may affect gene expression in rice were avoided in *NmDef02*, and the codons were optimized based on preferred codons in rice. During the synthesis, the original signal peptide was substituted by the *Dahlia merckii* antimicrobial peptide 1 (Dm-AMP1) which had successfully targeted to rice apoplast (Jha et al., 2008).

The plasmid p35SHyg contained the hygromycin phosphotransferase gene (*hpt*) as selectable marker, regulated by CaMV35S promoter.

Transformation Procedure

Disinfected mature seeds of indica rice (*Oryza sativa,* cv. J-104) were cultured, at 28 °C in the dark, on the callus induction medium containing N6 medium (Chu et al., 1975) supplemented with 2 mg/L 2.4-D, 30 g/L sucrose, 2.5 g/L phytagel, (pH 5.8). After 3 weeks, embryogenic calli with 3-4 mm diameter were co-transformed using a Biolistic PDS-1000/He (Bio-Rad, UK Ltd.). Particle bombardment, selection and regeneration of transformants were performed using the procedure described by Pérez-Bernal et al. (2017).

Primary co-transformants resistant to hygromycin were acclimated under natural conditions in a grow house protected by bird netting. For the inheritance study, there were selected only the individual plants that fulfilled the descriptive characters reported by Puldón et al. (2002) for the traditional J-104 rice cultivar in the maturation stage (Table 1). The seeds obtained from these plants were germinated on solid N6 culture medium (Chu et al., 1975) with 50 mg/L hygromycin. They were used for studying the segregation of the transgenes in plants of T1 progeny.

Southern blot in genomic DNA of plants from T1 progeny

Genomic DNA from 10 µg of non-transformed and T1 transgenic plants was digested with restriction enzyme EcoRV to confirm the insertion of *NmDef02* gene, electrophoresed on 1% agarose gel and blotted onto Hybond-N+ membrane (Amersham, Biosciences). DNA was fixed to the membrane 5 min in the UV

transilluminator. Hybridization probe (231 bp) was labeled with a non-radioactively detection system (DIG High Prime DNA Labeling and Detection Starter Kit II, Boehringer Mannheim) and the hybridization was performed as recommended by supplier.

Table 1. Descriptive characters established for the J-104 rice cultivar in the maturation stage.

Descriptive characters	Expert criteria for J-104 rice cultivar
Panicle length (cm)	26.7
Dry weight of 1000 seeds (g)	31.0
Seeds per panicle	127
Seed length (mm)	9.38
Seed width (mm)	2.6
Length/width ratio	3.60

Source: Catalog of Cuban rice varieties (Puldón et al., 2002)

Quantitative real-time PCR for *NmDef02* in individual T1 plants

Total RNA was extracted from young leaves of rice lines (T1 progeny) and non-transformed control using the RNeasy kit (Qiagen, USA), including an on-column DNAse treatment (Qiagen, USA) according to manufacturer's instructions. The cDNA was synthesized using an oligo-(dT) primer and Reverse transcriptase enzyme (Promega, USA) according to the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR) was conducted using a Rotor-Gene 3000 PCR machine (Corbett, Sydney, Australia) with the QuantiTect SYBR Green PCR kit (Qiagen, USA). *NmDef02* cDNA was amplified using the primers:

Forward:
5'-AAG CTT ATG CGT GAG TGC AAG GCT C-3'

Reverse:
5'-CTG CAG TTA GCA CTC GAA TAT AC-3'

This assay was used to measure the relative expression of *NmDef02* transcript levels, when compared to the constitutively expressed actin gene as an endogenous control. Real-time PCR conditions were as follows: initial 95 ºC denaturation step for 15 min, annealing for 30 s at 60 ºC, extension for 30 s at 72 ºC for 40 cycles. Analyses were carried out on the Rotor-Gene 3000 software (Corbett). All samples were triplicated and the threshold cycle values were quantified by calculating the means of normalized expression.

PCR analysis in four successive generations

Seeds germinated on solid N6 culture medium (Chu et al., 1975) without hygromycin, to ease the germination of marker-free plants. Plant DNA used for PCR analyses was extracted from young leaves using the protocol of Dellaporta et al. (1983). The PCR reactions were carried out in a total volume of 30 µL including 0.5 µg template DNA, 3.0 µL 10X buffer, 3.0 µL 10 mM dNTP, 3.0 µL 25 mM $MgCl_2$, 3*U* Taq DNA Polymerase (HeberZima, Cuba), 1.2 µL of each primer at 25 µM. DNA was denatured at 94 °C for 3 min followed by 30 amplification cycles (57 °C for 1 min, 72 °C for 1.5 min, 94°C for 1 min) and finally 10 min at 72 °C. The amplification products were checked by agarose gel electrophoresis. The expected product sizes were 410 bp and 630 bp, for the *NmDef02* and *hpt* genes, respectively.

Results and discussion

Culture of transformants

Many of the bombarded calli were found proliferating in the first round of selection on medium containing hygromycin. The number of proliferating calli was lesser in the subsequent rounds of selection. Shoot regeneration from hygromycin-resistant embryogenic calli was observed after 3-4 weeks of incubation on the regeneration medium. A total of 23 putative transformants were regenerated from calli selected on hygromycin-containing medium, but seven of these plants died on rooting medium with hygromycin. The use of selective agent in the rooting medium helps to reduce the number of non-transgenic plants surviving after selection rounds. A more extensive selection of calli on hygromycin-containing medium has not been recommended, since it could affect plant regeneration (Pérez-Bernal et al., 2017).

NmDef02 gene in plants from T1 progeny

Sixteen lines of primary co-transformants, considered as independent transformation events, grew under natural

conditions and were self-pollinated to obtain T1 progeny. For the transgene inheritance study, there were selected the lines 3, 6, 7 and 12 since they fulfilled all the descriptive characters reported by the experts for the J-104 rice cultivar in the maturation stage (Table 2). This selection was made with the main objective of eliminate the primary transformants with undesirable somaclonal variations.

Table 2. Evaluation of primary transgenic rice lines using the descriptive characters established for the J-104 rice cultivar in the maturation stage. Gray rows highlight lines that fulfill all descriptive characters.

Primary transgenic lines	Panicle lenght (cm)	Dry weight of 1000 seeds (g)	Seeds per panicle	Seed length (mm)	Seed width (mm)	Length/width ratio
1	26.44	31	133	9.28	2.63	3.52
2	26.02	30	121	9.35	2.63	3.55
3	26.7	31	127	9.38	2.60	3.60
4	23.81	30	107	9.42	2.87	3.28
5	24.50	29	89	9.54	2.84	3.36
6	26.7	31	127	9.38	2.60	3.60
7	26.7	31	127	9.38	2.60	3.60
8	23.37	28	96	9.10	2.82	3.22
9	26.70	27	120	9.15	2.59	3.53
10	22.94	29	89	9.33	2.90	3.22
11	23.70	29	90	9.10	2.77	3.28
12	26.7	31	127	9.38	2.60	3.60
13	23.00	31	96	9.45	2.91	3.25
14	26.78	30	126	9.38	2.83	3.31
15	22.10	30	104	9.45	2.60	3.63
16	26.81	28	121	9.30	2.64	3.52

The T1 generation plants of these selected lines germinated on solid culture medium without hygromycin. Southern blot analysis confirmed the hybridization with the *NmDef02* probe in the lines 6, 7 and 12 (Fig. 1A).

The relative expression of *NmDef02* mRNA was examined by qRT-PCR in these lines. As shown in Figure 1B, the lines displayed different expression levels of the *NmDef02* cDNA. The line 12 showed the highest relative expression of this defensin. Changes in *NmDef02* expression between lines could be attributed to different chromosomal location of the transgene and its interaction with nearby genes, as stated by Betts and colleagues (2019). These authors evaluated the transgene insertion sites in maize and soybean using both random and site-specific transgene integration and concluded that only specific sections of the genome are suitable for transgene insertion.

The *hpt* gene was detected by PCR in the evaluated lines (Fig. 1C). That indicated a 100% of co-integration of both transgenes in the three lines. Although the co-transformation molar ratio 4:1 favored the insertion of *NmDef02* over *hpt*, the 100% of co-integration could be explained since hygromycin B was used as a selective agent and it favored the selection of resistant specimens from callus phase until the last step of selection on rooting medium.

The frequency of co-integration of transgenes appears to vary in different species and also when different

plasmids are used. Chong-Pérez and colleagues (2013) demonstrated that co-integration of two transgenes in banana, using a Cre/lox system controlled by an embryo specific promoter, averages 70% and greatly depends on the DNA ratio in the plasmid mixture. The co-integration rate of linked and unlinked genes can also vary between different experiments using same plasmids and also under similar experimental conditions (Devi et al., 2012).

The lines 6, 7 and 12 were selected to continue the inheritance study in subsequent generation.

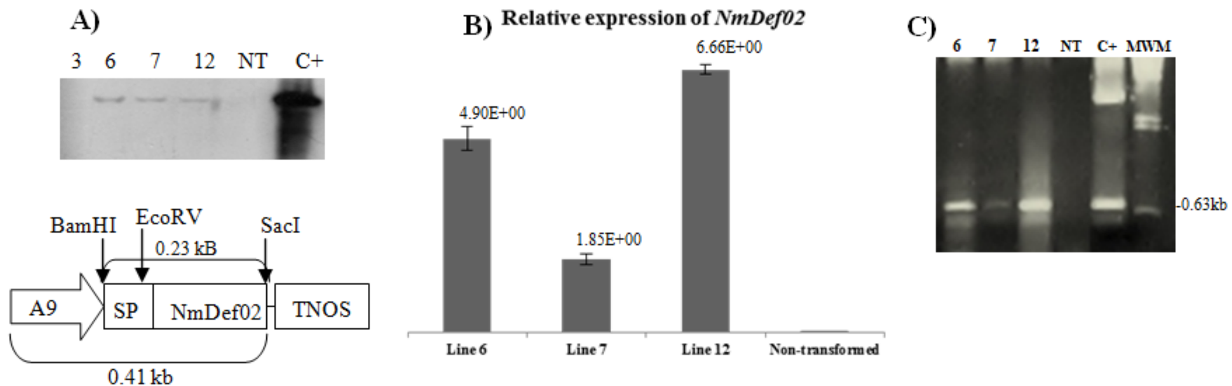


Fig. 1: Molecular analysis of transgenic rice plants from T1 progeny. **A)** Southern blot assay for the *NmDef02* gene. Hybridization probe was labeled with a non-radioactively detection system. Lanes: 3, 6, 7 and 12: transgenic lines; NT non-transgenic plant; C+: positive control (pA9NmDef02). **B)** Relative expression of *NmDef02* defensin gene evaluated by RT-PCR. Bars represent mean values of normalized expression and standard error from three replicates per line. **C)** Amplification by PCR of the 0.63 kb fragment corresponding to the *hpt* gene. Lanes: 6, 7 and 12: transgenic lines; NT: non-transgenic plant; C+: positive control (p35SHyg); MWM: molecular weight marker.

Transgenic analysis through three successive progenies

PCR analysis of segregating T2 progeny indicated the independent segregation of the *NmDef02* and *hpt*. It was detected two gene combinations in the evaluated plants: *NmDef02*(+)*hpt*(+) and *NmDef02*(+)*hpt*(-). All plants were found to carry the *NmDef02* gene (Fig. 2A), but in five of them the *hpt* gene was not detected by PCR (Fig. 2B), so they was marker-free plants harboring the gene of interest.

Generation of selection marker-free transgenic plants is a goal of plant breeding, since marker genes pose safety concerns regarding the expression of resistance marker genes that could cause antibiotic resistance in plant pathogens, potentially posing a threat to humans and animals (Xu et al., 2020). Therefore, it is crucial to develop transgenic plants with no selectable markers, and the independent transgene segregation after co-transformation is a suitable strategy for the compliment of this objective (Pérez-Bernal et al., 2017; Singh et al., 2019). One aspect that makes co-transformation advantageous is that it does not require additional genetic manipulation for removal of the selectable marker gene, as required for example in the Cre/loxP-based approach (Chong-Pérez et al., 2013).

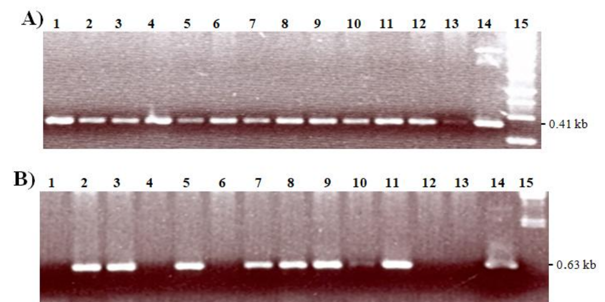


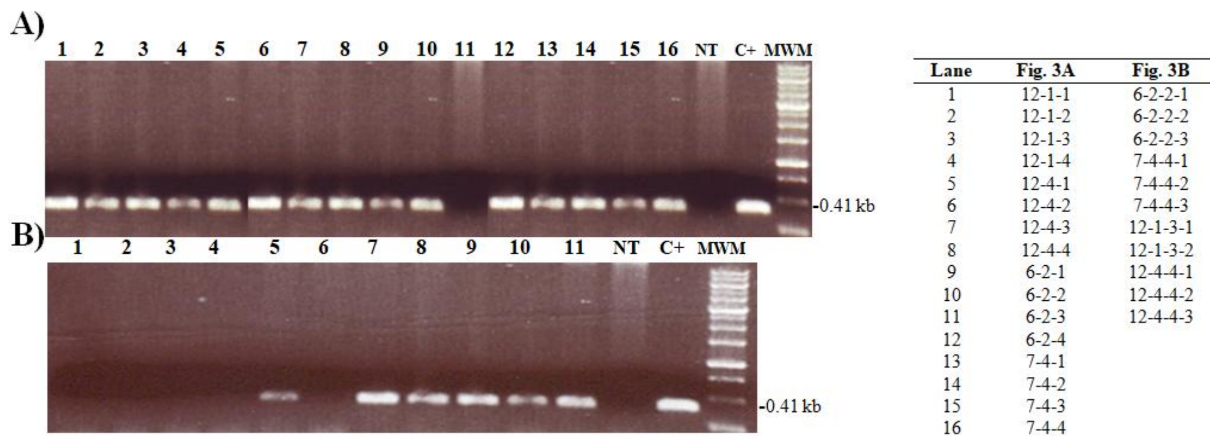
Fig. 2: PCR analyses in genomic DNA of transgenic rice plants from T2 progeny. **(A)** Amplification of the 0.41kb fragment corresponding to the *NmDef02* gene. **(B)** Amplification of the 0.63kb fragment corresponding to the *hpt* gene. Lanes 1-13: evaluated lines with the following consecutive identification (6-1, 6-2, 6-3, 6-4, 7-1, 7-2, 7-3, 7-4, 12-1, 12-2, 12-3, 12-4 and 12-5); Lane 14: positive control in each figure **(A)** pA9NmDef02 and **(B)** p35SHyg; Lane 15: molecular weight marker.

Feng et al. (2017) created marker-free drought-tolerant transgenic rice plants by biolistic co-transformation.

They discussed that using this transformation method it is difficult to separate the selection marker from the gene of interest since it is present in multiple copies. Consequently, a large number of progeny must be screened to detect a marker-free plant. Our results did not agree with the criteria of these authors, because we identified marker-free transgenic plants in a few lines of the second progeny without requiring an extensive inheritance screening. Applying a strategy similar to ours, Pan et al. (2020) obtained marker-free transgenic rice plants by co-transforming with the rice blast resistance gene Pi9 and the *hpt* marker. Rajadurai et al. (2018) developed insect-resistant and marker-free transgenic rice, by using the novel gene cry2AX1 in one cassette and the selectable marker gene in another. Both genes were co-integrated and then subjected to natural homozygous recombination / segregation to produce the marker-free transgenic lines.

As PCR analysis exhibited that the *NmDef02* transgene were stably inherited in T2 generation, the marker-free lines harboring the *NmDef02* (12-1, 12-4, 6-2, and 7-4) were elected to continue the inheritance study. Mature seeds were obtained successfully under natural conditions, and they were seeded on solid culture medium without hygromycin, to get the next progeny.

The results of the PCR in the plants from T3 progeny showed that in plant 6-2-3 the gene of interest was not detected (Fig. 3A). The lines 12-1-3, 12-4-4, 6-2-2 and 7-4-4 were chosen to continue the segregation study in T4 generation. Almost in the 50% of the evaluated plants from this last progeny the gene of interest was not found, a result that could suggest a sign of transgene instability, but this gene was detected in all plants of the line 12 (Fig. 3B). This transgenic line was considered a good transformation event because it fulfills the descriptive characters established for the traditional variety, it is a marker-free line since the T2 generation, it has the highest expression of *NmDef02* and this gene was detected across the four generations assessed.



Lane	Fig. 3A	Fig. 3B
1	12-1-1	6-2-2-1
2	12-1-2	6-2-2-2
3	12-1-3	6-2-2-3
4	12-1-4	7-4-4-1
5	12-4-1	7-4-4-2
6	12-4-2	7-4-4-3
7	12-4-3	12-1-3-1
8	12-4-4	12-1-3-2
9	6-2-1	12-4-4-1
10	6-2-2	12-4-4-2
11	6-2-3	12-4-4-3
12	6-2-4	
13	7-4-1	
14	7-4-2	
15	7-4-3	
16	7-4-4	

Fig. 3: Amplification by PCR of the 0.41kb fragment corresponding to the *NmDef02* in genomic DNA of transgenic rice plants from T3 **(A)** and T4 **(B)** progenies. Numbered lanes: Transgenic plants identified in the table attached to the figures; NT: non-transformed plant; C+ positive control (pA9NmDef02); MWM: molecular weight marker.

The loss of the gene of interest in three of the four lines evaluated in the T4 progeny may be due to some putative mechanisms that affect the transgene inheritance, for example, the nature of the transgene itself, the linkage between the transgene integration and target site, multiple unlinked transgene integrations (Michno et al., 2020). In some cases the transgenic locus has not been stably inherited or the transgenes are inactivated if the integration occurs in the repeat-sequence regions of heterochromatin (Yin et al., 2004). Tizaoui and Kchouk (2012) explained that endogenous and exogenous stimuli, such as transgenes newly integrated into the genome, can increase the frequency of crossing-over. This means an alteration in a process that occurs naturally in plants and whose main function is to generate new genetic combinations.

Unstable inheritance is also accounted by transgene deletions, which suggesting that the foreign DNA could be unstably integrated or absent from the gametes (Yin et al., 2004). The segregation distortion of transgenes among T1 progeny has been commonly observed, and several hypotheses have explained this event, such as T0 chimerism, multiple independently assorting

insertion loci and transgene silencing induced by multiple transgene copies or DNA rearrangements (Hensel et al., 2012). Hiei et al. (1994) described atypical segregation patterns of GUS expression in the progenies of rice transformants. It seemed that the chimerism in the T0 generation (primary transformants) had an effect on the stability of the transgene. These results differ from ours, since the instability of the gene of interest was evidenced from the third generation of transgenic rice plants.

Conclusions

Analysis of the inheritance of two transgenes over four successive generations of rice has provided information concerning the transgenic population. The co-transformation strategy by biolistic method with the *NmDef02* as gene of interest and the selection marker guarantees the obtaining of marker-free transgenic rice lines. The relative expression levels of *NmDef02* are demonstrated, but the inheritance of this defensin gene shows signs of instability from the third generation of plants. The molecular analysis carried out in this work does not allow demonstrating the mechanisms that influenced the instability in the inheritance of the gene of interest. Finding an answer to this behavior will be the goal of future research.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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