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Research Article

THE FIRST SUCCESS IN UTILIZING THE *RUBY* REPORTER GENE FOR RICE TRANSFORMATION IN VIETNAM

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ABSTRACT

To date, several reporter genes have been developed, such as *GFP, GUS,* and *luciferase*. However, *GFP* and *luciferase* genes are costly and require modern equipment, while GUS requires sample destruction. To overcome these limitations, the *RUBY* gene is considered a promising alternative. *RUBY* is an artificial open-reading frame, encoding CYP76AD1, L-DOPA 4,5-dioxygenase, and glucosyltransferase, which are key enzymes involved in the biosynthesis of betalain, which displays a red-colored pigment, providing a visual marker for transformation events. In this study, the *RUBY* gene was successfully transformed into the model rice variety Kitaake via the *Agrobacterium*-mediated method. The activity of the *RUBY* gene was visually detected by red spots in calli and somatic embryos through the rice transformation and regeneration procedure. Moreover, the presence and expression of this reporter gene were confirmed by PCR and the red coloration in the regenerated rice lines. The *RUBY* gene was stably expressed in transgenic rice lines under both *in vitro* and greenhouse conditions and was heritable in the T1 generation, without exhibiting any adverse effects on plant growth and development. This result demonstrates the potential of *RUBY* as a visual selection marker for rice transformation in Vietnam and opens the door to further applications in other important crops.

Keywords: Betalains, Kitaake, reporter gene, rice, RUBY.

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INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important staple food crops among cereals (Verma and Srivastav, 2020). As the global population is projected to reach 9.7 billion by 2050 (World Population Prospects 2024), the demand for food production, particularly rice, is expected to increase significantly. However, rice cultivation faces critical challenges due to climate change and pest and disease threats. Addressing these challenges, genetic improvement of rice has become a vital strategy to enhance crop productivity and resilience. Traditional and advanced techniques, including hybrid breeding (Miah *et al*., 1996), protoplast fusion (Fish *et al*., 1988), and mutagenesis via UV or chemical agents (Davey and Anthony, 2010) have yielded significant achievements. *Agrobacterium tumefaciens (A. tumefaciens)*-mediated transformation has been seen as a preferred approach for plant genetic engineering and crop improvement (Slamet-Loedin *et al*., 2014). Various transgenic events have been commercialized worldwide and have significantly contributed to global food security.

Reporter genes are valuable tools in plant transformation, allowing researchers to monitor transgene expression and select transformed tissues effectively. Among commonly used reporter genes such as GFP (Green Fluorescent Protein), GUS (β-glucuronidase), and luciferase, each offers distinct advantages but also has notable limitations. For instance, while GFP is relatively simple to use, it requires UV or blue light sources for fluorescence observation (Chalfie *et al*., 1994), which limits its practicality in routine observation. One of the most common reporter genes for plant transformation is the GUS gene, which also involves invasive procedures and destructive sampling, making the transformed tissue unusable for further experiments (Thao *et al.*, 2022; Jefferson *et al*., 1987). Meanwhile, luciferase, though highly sensitive, requires a specialized camera system and costly substrates, such as luciferin, increasing the cost and complexity of experiments (Ow *et al*., 1986). Taken together, these drawbacks highlight the need for cost-effective, non-invasive, and efficient reporter systems.

In 2020, He and colleagues introduced a novel reporter gene system named *RUBY*, which has the potential to overcome the limitations mentioned above (He *et al*., 2020). *RUBY* is an artificial open reading frame that encodes three key enzymes, CYP76AD1, L-DOPA 4,5-dioxygenase, and glucosyltransferase, which are involved in the biosynthetic pathway converting tyrosine into betalain, a naturally occurring red pigment that can be easily observed with the naked eye without the need for complex equipment or chemical treatments. This pathway requires the coordinated expression of all three genes to successfully produce betalain. The process begins with CYP76AD1, a cytochrome P450 monooxygenase, which hydroxylates tyrosine to form L-3,4-dihydroxyphenylalanine (L-DOPA) which is then oxidized to cyclo-DOPA. Subsequently, L-DOPA is also oxidized and spontaneously cyclized to generate betalamic acid. These two intermediates, cyclo-DOPA and betalamic acid, then condense non-enzymatically to form betanidin. Finally, the glucosyltransferase enzyme glycosylates betanidin, producing betalain, the red pigment. Through this traceable biosynthetic route, the *RUBY* gene offers researchers a

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visual, non-invasive, and cost-effective approach for monitoring transgene expression.

Recently, multiple studies have demonstrated and documented the effectiveness of *RUBY* as a marker in plant transformation. Notably, Lee and co-workers developed new T-DNA binary vectors harboring the *RUBY* reporter gene, which improved the efficiency of maize transformation and targeted mutagenesis (Lee *et al*., 2023). Similarly, Wang and colleagues used the *RUBY* reporter to accurately identify haploid maize by enabling clear betalain-based color distinction (Wang *et al*., 2023). In rice, He and colleagues successfully utilized the *RUBY* gene as a visual reporter during *Agrobacterium*-mediated transformation (He *et al*., 2020). The *RUBY* gene was stably integrated and expressed in both calli and regenerated rice plants, enablingclear visualization of various transformation stages. However, to date, no published report on the application of the *RUBY* gene as a visual marker for plant transformation in Vietnam. Motivated by the growing demand for plant transformation research and based on the available scientific evidence demonstrating the effectiveness of the *RUBY* gene, we conducted a study to evaluate *RUBY* as a visual marker for *Agrobacterium*-mediated rice transformation in Vietnam. The primary objective of this study was to assess the feasibility and effectiveness of the *RUBY* reporter gene in identifying successful transformation events throughout the transformation process, plant regeneration, and its heritability in subsequent generations. This success provides a foundation for expanding the application of the *RUBY* reporter gene to transformation in various local rice cultivars and other important crop species in Vietnam.

MATERIALS AND METHODS

Materials

Mature seeds of rice variety Kitaake were provided by the Plant Cell Biotechnology Laboratory, Institute of Biology, Vietnam Academy of Science and Technology. *A. tumefaciens* strain AGL1 harbouring the 35S:RUBY vector (Addgene plasmid #160908), provided as a gift from Yunde Zhao's lab (Figure 1). Specific primers were synthesized by PHUSA Biochemistry (Can Tho, Vietnam), which were utilized to confirm the presence of transgenes in transgenic rice lines (Used primer sequences are listed in Table 1). The compositions of the culture media used in this study are detailed in Table 2. Basal medium components, including N6 macro salts, MS salts and B5 vitamins, were supplied by PlantMedia (Ohio, USA). Amino acids, sugars, and components for bacterial culture media were obtained from BioBasic (Ontario, Canada). Phytohormones and antibiotics were purchased from Sigma-Aldrich (Missouri, USA).

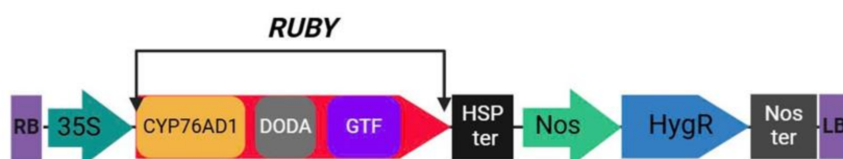

Figure 1. The 35S: RUBY construct for rice transformation. RB: Right Border; LB: Left Border; 35S: Cauliflower Mosaic Virus 35S promoter; CYP76AD1, DODA, GTF are three genes involved in the betalain biosynthesis, which are fused within a single open reading frame named *RUBY*; HSP ter: heat shock protein terminator; HygR: hygromycin resistance gene; Nos: nopaline synthase promoter; Nos ter: nopaline synthase promoter terminator.

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Table 1. List of primers used in this study

Primers	Nucleotide sequence (5'–3')	PCR products (bp)
hptII-F	TTTATCGGCACTTTGCATCG	614
hptII-R	GAGCATATACGCCCGGAGTC	
CYP-F	CCAAGATTCACGGCCCACTC	631
CYP-R	TCGGTGGTGGTGGTAGTTGT	
DODA-F	AGAAGATTTTCTCCAAGAAGCCGA	394
DODA-R	ATAATGAGCACGCCATCATTCTTG	
GTF-F	AGATGATGGAACTCGCTGCT	518
GTF-R	GGAGCCCTTCAGCTCTTTTT	

Methods

Agrobacterium-mediated transformation

A. tumefaciens strain AGL1 carrying the 35S:RUBY construct with hygromycin resistance gene *hptII* was cultured on YEP solid medium supplemented with 100 mg/L spectinomycin and 25 mg/L rifampicin. A procedure for *Agrobacterium*-mediated transformation of the Kitaake variety was modified and improved from the report by Sallaud and colleagues (Sallaud *et al*., 2003). Briefly, mature seeds were surface-sterilized and cultured on NB medium for embryogenic callus induction. The resulting calli were subsequently excised from the seeds and transferred to fresh NB medium for further development until they reached a stage suitable for transformation. After 10 days, the calli were immersed in a co-cultivation liquid (CCL) containing *A. tumefaciens* at a density of OD_{600} = 0.1 for 20 min. Then, the calli were blotted and transferred to a co-cultivation solid medium (CCS). After a 3-day co-cultivation, the calli were subcultured on selection medium R2S supplemented with 50 mg/L hygromycin and appropriate antibiotics to eliminate the growth of *Agrobacterium*. One week later, good calli were transferred to NBS medium to continue the selection process. Opaque and yellowish calli exhibiting hygromycin resistance were transferred to pre-regeneration medium (PRAG). Sub-sequently, they were moved to regeneration medium (RN) under light conditions. Shoots regenerating from hygromycin-resistant calli were transferred to Murashige and Skoog medium to promote further shoot elongation and root development.

DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was isolated from the young leaves of T0 transgenic rice lines (the initial generation of transformed plants) using a modified CTAB method, developed by Doyle and Doyle (1990). The extracted DNA served as a template for polymerase chain reaction (PCR) to verify the presence of three genes associated with betalain biosynthesis in the T-DNA, along with the *hptII* selection marker gene (Linh *et al*., 2023). The PCR was performed under the following thermal cycling conditions: initial denaturation at 95°C for

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5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. A final elongation step was carried out at 72°C for 5 min to ensure complete amplification of target DNA fragments. Following amplification, PCR products were assayed by electrophoresis on 1.5% agarose gel.

Growth in greenhouse conditions

To investigate the effect of the *RUBY* gene on the inheritance of rice to the next generation, transgenic *RUBY* lines and wild-type (WT) Kitaake control plants were cultivated under greenhouse conditions. The two-week-old *in vitro*-grown plants on rooting media were placed in plastic pots (20 cm in diameter by 20 cm in height) filled with water-soaked soil (80% alluvial soil and 20% TRiBAT® compost mixture) in a greenhouse setting. After 10 weeks, T1 seeds (the first generation of T0 plants) of WT and *RUBY* transgenic plants were collected to evaluate the inheritance of the T1 generation.

Table 2. Compositions of different media used in the study

Medium	Label	Compositions
Stock NB	NB_0	N6 major salt, Fe-EDTA (Chu *et al.*, 1975), B5 Minor salt, B5 Vitamins (Gamborg *et al.*, 1968), 500 mg/L proline, 500 mg/L glutamine, 100 mg/L myo-inositol, 300 mg/L casein hydrolysate, 30 g/L sucrose, 3 g/L phytagel, pH 5.8 (Li *et al.*, 1993)
Callus induction	NB	NB_0, 2.5 mg/L 2,4-D, pH 5.8
Stock R2	$R2_0$	R2 major salt, R2 minor salt, R2 Fe-EDTA, R2 Vitamins (Ohira *et al.*, 1973).
Co-cultivation liquid	CCL	$R2_0$, 10 g/L glucose, 2.5 mg/L 2,4-D, 100 µM AS, pH 5.2
Co-cultivation solid	CCS	$R2_0$, 10 g/L glucose, 2.5 mg/L 2,4-D, 100 µM AS, 3 g/L phytagel, pH 5.2
First selection	R2S	$R2_0$, 30 g/L sucrose, 2.5 mg/L 2,4-D, 500 mg/L cefotaxime (cef), 50 mg/L timentin, 50 mg/L hygromycin (hyg), 3 g/L phytagel, pH 5.8
Second selection	NBS	NB_0, 2.5 mg/L 2,4-D, 400 mg/L cef, 30 mg/L timentin, 50 mg/L hyg, pH 5.8
Pre-regeneration	PRAG	NB_0, 2 mg/L BAP, 1 mg/L NAA, 5 mg/L ABA, 200 mg/L cef, 30 mg/L timentin, 50 mg/L hyg, pH 5.8
Regeneration	RN	NB_0, 3 mg/L BAP, 0.5 mg/L NAA, 200 mg/L cef, 50 mg/L hyg, pH 5.8
Rooting	MS	MS (Murashige and Skoog, 1962), 30 g/L sucrose, 200 mg/L cef, 50 mg/L hyg, pH 5.8
Bacterial culture	YEP	10 g/L yeast extract, 10 g/L bacto peptone, 5 g/L NaCl, 15 g/L bacto agar, pH 7.0

RESULT AND DISCUSSION

Transformation and regeneration of T0 transgenic rice lines

To generate transgenic rice lines carrying the *RUBY* gene, the 35S: RUBY construct was transformed into Kitaake via *A. tumefaciens* (Figure 2). The red pigment was first

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detected at 2 weeks after transformation on the second selection medium (Figure 2E), which was later than in maize or soybean transformation, where betalain accumulation was observed from very early stages, only 2 to 3 days after transformation (Lee *et al*., 2023; Chen *et al*., 2024).

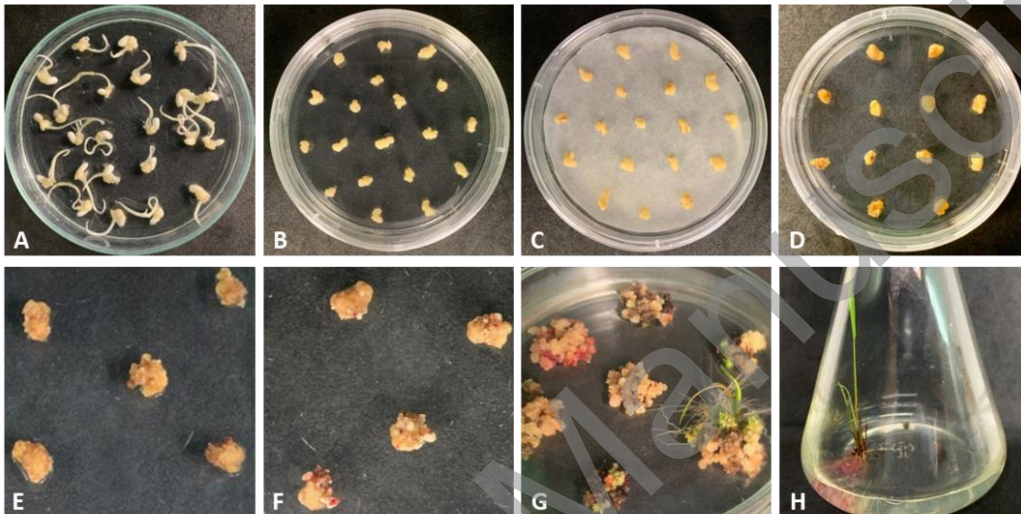

Figure 2. *A. tumefaciens*-mediated transformation procedure for Kitaake. A: Callus induction; B: Sub-cultured callus; C: Co-culture; D: Callus on the first selection medium; E: Callus on the second selection medium; F: Pre-regeneration; G: Regeneration; H: Rooting and developing transgenic shoots

From the second selection stage to the regeneration stage, the expansion of red calluses indicated the proliferation of somatic cells containing the *RUBY* gene. The number of calli expressing the *RUBY* gene also increased in the pre-regeneration and regeneration stages (Figure 2F-G). The expression of the *RUBY* gene allowed us to visually distinguish transformed calli from non-transformed calli, and to distinguish calli with better expression of the transgenic genes among hygromycin-resistant calli in the same explant. These results showed that the *RUBY* reporter could serve as an excellent visual marker during rice transformation. In total, 13 red-pigmented calli with shoot regeneration potential were obtained. Each callus produced from 1 to 12 shoots capable of rooting on the medium supplemented with hygromycin (Figure 2H). For each callus, one rice plant was randomly selected for gene analysis and further studies.

Confirmation of the presence of transgenes in T0 regenerated rice lines

The transgenic lines were confirmed through both visual observation of *RUBY* gene expression and molecular detection of the transgenes. Morphologically, the most distinct and easily recognizable trait was the appearance of the characteristic red pigment of betalain, the end product of the metabolic pathway regulated by the *RUBY* gene. Among tissues, the root exhibited the most prominent coloration, enabling clear differentiation between transgenic plants and non-transgenic plants (Figure 3A). This is attributed to the absence of chlorophyll and other masking pigments in root tissues. In some successfully regenerated transgenic lines, red pigmentation was also observed in

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other plant organs such as stems and leaves. In different stages of plant regeneration, red coloration in these organs may be masked by the dominant presence of chlorophyll, particularly in mature leaves. However, the red pigmentation was visually detectable in young leaves (Figure 3B).

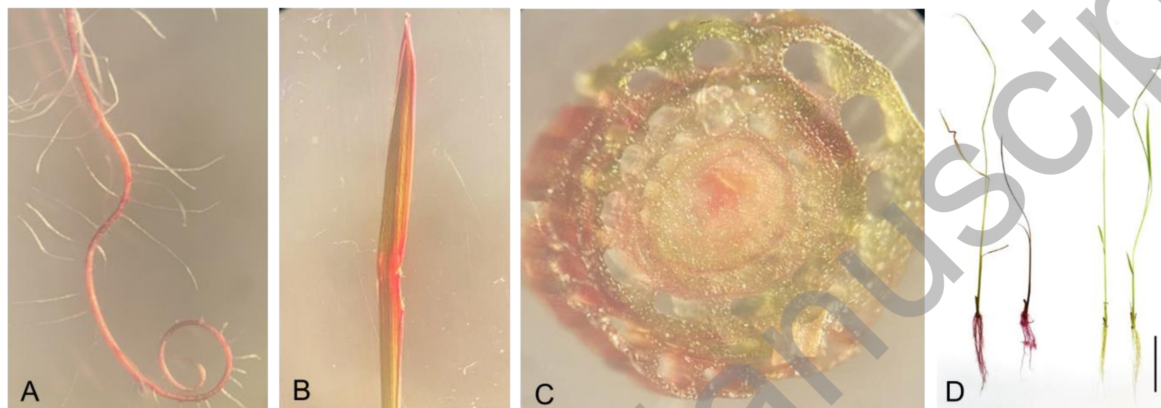

Figure 3. *RUBY* expression in T0 transgenic rice plants. A: Root. B: Stem. C: Leaf. D: Total plant. Scale bar = 5 cm.

Similar to the leaves, stems also displayed betalain pigmentation, though the coloration was sometimes obscured by outer covering tissues. To confirm endogenous expression, cross-sectional analysis of stems revealed the red pigmentation in internal tissues, particularly parenchyma and vascular tissues, reflecting *RUBY* gene activity in meristematic and vascular regions (Figure 3C). Notably, in some transgenic individuals, lateral tillers derived from the primary plant also maintained and expressed red pigmentation in all of their parts, demonstrating the stable expression of the *RUBY* gene (Figure 3D). These findings are consistent with the observations of He and colleagues, in which red pigmentation was visibly observed in the leaves and roots of regenerated rice plants, indicating betalain biosynthesis in vegetative tissues (He *et al.*, 2020). Similar patterns of betalain pigment accumulation have also been reported in other crop species. Sun and co-workers documented pronounced betalain accumulation in immature leaves and shoots, which was visually observed by the naked eye (Sun *et al.*, 2023). Likewise, in maize, Lee and partners observed betalain expression as stripes or uniform dark purple coloration in the leaves, as well as the vascular bundle tissues of the stem (Lee *et al.*, 2023).

Within the designed T-DNA region, an expressed cassette of the *hptII* gene encoding phosphotransferase was constructed. This enzyme phosphorylates hygromycin B, thereby inactivating the antibiotic (Blochlinger and Diggelmann, 1984). As a result, transgenic plants harboring the transgene exhibit resistance to hygromycin. To confirm the presence of the hygromycin resistance gene *(hptII)* in 13 regenerated rice lines, PCR was conducted using a specific primer targeting the *hptII* gene (as listed in Table 1). PCR products were analysed via electrophoresis on a 1.5% agarose gel. An expected DNA band of 614 bp, corresponding to the expected size of the *hptII* fragment, was observed in all 13 regenerated lines, while no PCR product

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appeared in the WT control (Figure 4). These results confirm the successful integration of the *hptII* selectable marker into the genomes of all regenerated rice lines.

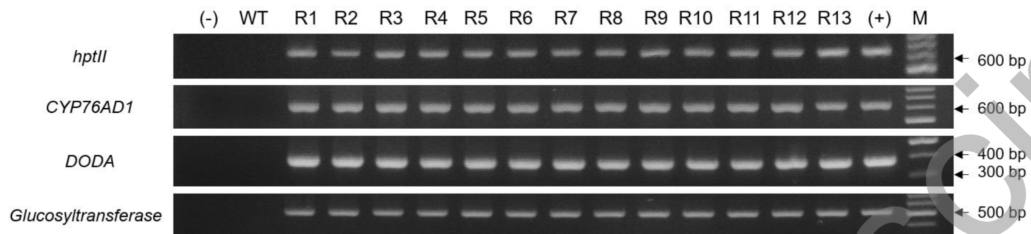

Figure 4. PCR confirmation of the presence of *hptII* gene and three genes related to Betalain biosynthesis *(CYP76AD1, DODA* and *Glucosyltransferase*). WT: Kitaake wild-type plant, R1-R13: T0 Transgenic rice lines, (+): Positive control, (-): Negative control, M: GeneRuler 100 bp DNA Ladder (Thermo Scientific).

However, the presence of the *hptII* gene alone does not provide sufficient evidence for the complete integration of the betalain biosynthetic genes, which is required for phenotypic expression of red pigmentation. As mentioned above, betalain expression is the outcome of the coordinated activities of three functional genes, including *CYP76AD1*, *DODA*, and *Glucosyl-transferase*. Therefore, we performed PCR for each gene using specific primer pairs to verify the stable incorporation of the whole betalain biosynthetic genes into the rice genome (Table 1). The PCR-gel running results revealed three expected products of 631 bp (*CYP76AD1*), 394 bp (*DODA*), and 518 bp (*glucosyltransferase*) in all 13 transgenic lines, while no corresponding band was detected in the WT samples. These findings conclusively demonstrate the successful integration of the full betalain biosynthetic gene into the genomes of the transgenic rice lines, thus providing the molecular foundation for the observable red pigmentation phenotype in plant tissues.

Expression of RUBY in T0 rice plants under greenhouse conditions

To evaluate the effect of the *RUBY* gene on the growth of rice plants as well as to evaluate the stable expression of the *RUBY* gene in transgenic rice lines, Kitaake and RUBY transgenic rice lines were grown in greenhouse conditions. Our results showed no difference in growth between the control WT Kitaake and *RUBY* transgenic rice lines (Figure 5). In the report by He *et al*. (2020), the *RUBY* gene was transferred into Xiaowei^{NIP25} rice, which has a dwarf phenotype. Consequently, all generated *RUBY* transgenic lines also displayed dwarfism, and no evaluation of the gene's effect on rice morphology had been reported. Another study by Lee *et al*. (2023) on *RUBY* transformation in maize showed that the transgenic maize plants had extensive red pigmentation along with stunted growth (Lee *et al*., 2023). In our current study, the T0 transgenic *RUBY* rice lines had similar phenotypes to the WT Kitaake plants. However, further assessments at different generations need to be performed to evaluate the effects of *RUBY* gene overexpression on rice growth and development. This is the first success in utilizing the *RUBY* gene for rice transformation in Vietnam and proves the potential of the *RUBY* gene as a sufficient reporter gene for the transformation of other important crops.

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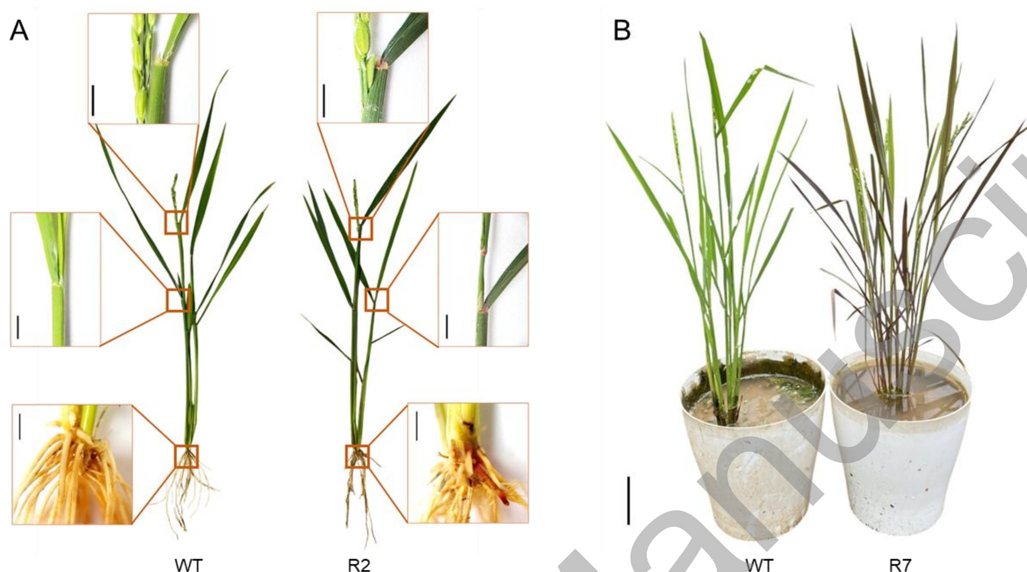

Figure 5. Expression of *RUBY* in 2-month-old wild-type plant and T0 transgenic rice line under greenhouse conditions. A: WT and T0 *RUBY* transgenic rice line 2. Scale bar = 0.5 cm. B: WT and T0 *RUBY* transgenic rice line 7. Scale bar = 15 cm.

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The *RUBY* gene served as an excellent visible marker for monitoring gene expression in transgenic rice. We observed stable expression of the *RUBY* gene in transgenic rice lines under both *in vitro* and greenhouse conditions. Under *in vitro* conditions, transgenic rice lines showed clear red betalain pigments in most parts of the plant (Figure 3). Under greenhouse conditions, transgenic lines could still be distinguished from WT plants by betalain accumulation. Different transgenic lines showed varying levels of betalain accumulation, ranging from red pigmentation in specific tissues to dark purple coloration across entire plants. In most cases, betalain was observed only in specific tissues such as ligules or roots (Figure 5A). Only a few lines displayed strong *RUBY* expression, with red pigmentation present throughout the entire plant (Figure 5B). No red pigmentation was observed in WT plants (Figure 5). These findings are consistent with previous studies by He *et al*. (2020) in rice and Chen *et al*. (2024) in soybean. He *et al.* (2020) reported *RUBY* expression throughout the entire Xiaowei^{NIP25} rice plant at one month of age under field conditions (He *et al*., 2020). Similarly, Chen *et al.* (2024) documented variation in betalain accumulation among different transgenic soybean lines (Chen *et al*., 2024). Our results confirm the stable expression of the *RUBY* gene across different environmental conditions. *RUBY* expression was observed at all developmental stages of transgenic rice, from callus tissue and *in vitro* plantlets to mature plants in the greenhouse.

Inheritance of the RUBY gene in T1 seed

After 10 weeks under greenhouse conditions, T1 seeds from transgenic rice lines and WT plants were harvested. The difference in husk color was noticeable: transgenic lines exhibited a slightly reddish-brown husk that

could be visually distinguished, whereas WT seeds retained their characteristic bright yellow color (Figure 6A). This pigmentation was even more pronounced in immature seeds, allowing early visual discrimination between transgenic and non-transgenic seeds. This phenotypic trait not only facilitated rapid screening but also provided a preliminary indication of rice color. Upon removal of the husk, T1 transgenic seeds displayed a distinctly dark red bran layer, clearly different from the ivory-white bran layer of WT seeds (Figure 6B). A similar result was observed in cross-sections of the seeds, where T1 transgenic seeds exhibited red-colored endosperm, which was easily distinguishable from the ivory-white endosperm in WT seeds (Figure 6C). These direct observations confirmed the stable inheritance of the *RUBY* gene expression from the T0 to the T1 generation.

Figure 6. *RUBY* expression in T1 transgenic rice seeds. A: Rice seeds with husk. B: Rice seed. C: Cross-section of rice seeds. Scale bar = 0.5 cm.

The application of the *RUBY* reporter gene in plant transformation has emerged as a new research direction in recent years, following the study by He *et al.* (2020). Notably, that study remains the only one to date that reported the use of the *RUBY* gene in rice transformation, limited to the T0 generation of the Xiaowei^{NIP25} variety. In this study, we demonstrated the heritability of the *RUBY* transgene in the Kitaake rice variety. The presence of the transgene in T1 seeds could be visually identified without genotyping, accelerating the selection of positive transgenic lines and reducing reliance on molecular assays such as PCR and electrophoresis. Our findings are strongly supported by recent studies in other crops. In soybean, Chen *et al.* (2024) demonstrated that the purple pigmentation was not only visible at early developmental stages (cotyledon emergence) but was also stably inherited across generations. The presence of the transgene in progeny could be readily distinguished based on seed color. Similarly, Lee *et al.* (2023) showed that in maize, betalain accumulation was consistently observed in various tissues, including T1 seeds. These results, observed in both monocots and dicots, provide compelling evidence for the stable transmission of the *RUBY* gene across generations. Furthermore, by integrating a *RUBY* expression cassette into a CRISPR/Cas9 vector targeting *GmWaxy*, Chen *et al*. (2024) reported that purple T1 seedlings displayed high editing efficiency at the target locus, with up to 66.7% exhibiting homozygous mutations. In contrast, the majority of green T1 seedlings retained the WT *GmWaxy* sequence. These findings demonstrate that *RUBY* is a reliable visual marker that greatly facilitates the screening and selection of genome-edited plants.

CONCLUSION

In this study, the *RUBY* reporter gene was successfully used in rice transformation via

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Agrobacterium-mediated transformation. PCR, hygromycin selection, and visual observation of pigment accumulation confirmed the presence and activities of transgenes. The red pigment of betalain was visually detected on rice calli at two weeks post-infection with *A. tumefaciens*. The *RUBY* gene was stably expressed in transgenic rice lines under both *in vitro* and greenhouse conditions. Notably, RUBY transgenic plants showed no growth differences compared to WT plants and produced T1 seeds with distinct red coloration visible to the naked eye. These findings indicate the *RUBY* gene can serve as an excellent marker for rice transformation, offering a reliable, non-destructive, and cost-efficient approach for identifying successfully transformed tissues.

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REFERENCES

Blochlinger, K., and Diggelmann, H. (1984). Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. *Molecular and Cellular Biology, 4* (12), 2929-2931. https://doi.org/10.1128/mcb.4.12.2929-2931. 1984

Phuong Thao, B., Thi Linh, N., Van Manh, N., Khanh Linh, L., Hoang Ha, C., Tien Phat, D., & Bich Ngoc, P. (2022). Optimization of Agrobacterium-mediated transformation procedure for an indica rice variety-Khang Dan 18. *Vietnam Journal of Biotechnology*, *20*(1), 53-62. https://doi.org/10.15625/1811-4989/172 00

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green Fluorescent Protein as a Marker for Gene Expression. *Science, 263*(5148), 802-805. https://doi.org/10.1126/science.8303295

Chen, L., Cai, Y., Liu, X., Yao, W., Wu, S., and Hou, W. (2024). The RUBY reporter for visual selection in soybean genome editing. *aBIOTECH, 5*(2), 209-213. https://doi.org/10.1007/s42994-024-00148-6

Chu, C.C., Chu, W.C., San, S.C., and Chen, H. (1975). Establishment of an efficient medium for another culture of rice through comparative experiments on the nitrogen sources. *Scientia Sinica, 18*(5), 659-668. https://doi.org/10.1360/ya1975-18-5-659

Davey, M.R., and Anthony, P. (2010). *Plant Cell Culture: Essential Methods* (1st ed.). Wiley, https://doi.org/10.1002/9780470686522

Doyle, J.J. and J.L. Doyle (1990). Isolation of plant DNA from fresh tissue. *Focus,* 12:13-15.

Fish, N., Lindsey, K., and Jones, M.G.K. (1988). Plant Protoplast Fusion. *Methods in molecular biology (Clifton, N.J.)*, 4, 481-498. https://doi.org/10.1385/0-89603-127-6:481

Gamborg, O.L., Miller, R.A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research, 50*(5), 151-158. https://doi.org/10.1016/0014-4827(68)90403-5

He, Y., Zhang, T., Sun, H., Zhan, H., and Zhao, Y. (2020). A reporter for noninvasively monitoring gene expression and plant transformation. *Horticulture Research*, *7*(1), 152. https://doi.org/10.1038/s41438-020-00390-1

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. (1987). GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal, 6*(13), 3901-3907. https://doi.org/10.1002/j.1460-2075.1987.tb02730.x

Khanh Linh, L., Van Doai, N., Thi Linh, N., Quyen, P., Hoang Ha, C., Bich Ngoc, P., and

Accepted Manuscript

Tien Phat, D. (2023). Development of CRISPR/Cas9 systems to induce targeted mutations in the promoter region of the OsSRFP1 gene in rice. *Vietnam Journal of Biotechnology*, *21*(3), 463-475. https://doi.org/10.15625/1811-4989/19532

Lee, K., Kang, M., Ji, Q., Grosic, S., and Wang, K. (2023). New T-DNA binary vectors with *NptII* selection and *RUBY* reporter for efficient maize transformation and targeted mutagenesis. *Plant Physiology, 192* (4), 2598-2603. https://doi.org/10.1093/plphys/kiad231.

Li, L., Qu, R., De Kochko, A., Fauquet, C., and Beachy, R.N. (1993). An improved rice transformation system using the biolistic method. *Plant Cell Reports, 12*(5), 250-255. https://doi.org/10.1007/BF00237129

Miah, M.A.A., Pathan, M.S., and Quayum, H.A. (1996). Production of salt tolerant rice breeding line via doubled haploid. *Euphytica*, *91*(3), 285-288. https://doi.org/10.1007/BF00033089

Murashige, T., and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum, 15*(3), 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Ohira, K., Ojima, K., and Fujiwara, A. (1973). Studies on the nutrition of rice cell culture I. A simple, defined medium for rapid growth in suspension culture. *Plant Cell Physiology, 14*(6), 1113-1121. https://doi.org/10.1093/oxfordjournals.pcp.a074950

Ow, D.W., Wood, K.V., DeLuca, M., De Wet, J.R., Helinski, D.R., and Howell, S.H. (1986). Transient and Stable Expression of the Firefly Luciferase Gene in Plant Cells and Transgenic Plants. *Science, 234*(4778), 856-859. https://doi.org/10.1126/science.234.4778.856

Sallaud, C., Meynard, D., Van Boxtel, J., Gay, C., Bès, M., Brizard, J.P., *et al.* (2003). Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. *Theoretical and Applied Genetics, 106*(8), 1396-1408. https://doi.org/10.1007/s00122-002-1184-x

Slamet-Loedin, I.H., Chadha-Mohanty, P., and Torrizo, L. (2014). Agrobacterium-Mediated Transformation: Rice Transformation, in: Henry, R.J., Furtado, A. (Eds.), Cereal Genomics, *Methods in Molecular Biology (Clifton, N.J.),* 1099, 261-271. https://doi.org/10.1007/978-1-62703-715-0_21

Sun, H., Wang, S., Zhu, C., Yang, K., Liu, Y., and Gao, Z. (2023). A new biotechnology for *in-planta* gene editing and its application in promoting flavonoid biosynthesis in bamboo leaves. *Plant Methods 19*(1), 20. https://doi.org/10.1186/s13007-023-00993-4

Verma, D.K., and Srivastav, P.P. (2020). Bioactive compounds of rice (*Oryza sativa* L.): Review on paradigm and its potential benefit in human health. *Trends in Food Science & Technology*, 97, 355-365. https://doi.org/10.1016/j.tifs.2020.01.007

Wang, D., Zhong, Y., Feng, B., Qi, X., Yan, T., Liu, J., *et al.* (2023). The *RUBY* reporter enables efficient haploid identification in maize and tomato. *Plant Biotechnology Journal, 21*(8), 1707-1715. https://doi.org/10.1111/pbi.14071.

Accepted Manuscript