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Transgenic Note

Highly efficient visual selection of transgenic rice plants using green fluorescent protein or anthocyanin synthetic genes

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Abstract Visual selection enables transformation in plants that are sensitive to the stress of antibiotic selection. Furthermore, we proposed a method of isolating transformed cells in which the gene of interest is expressed at high level using visual selection. However, visual selection was inefficient and laborious because of the technical difficulties involved in clonal propagation of transformed calli selected using GFP (9.4–20.5% in the model *japonica* rice variety Nipponbare in our previous report). Very recently, we have discovered that an *indica* model cultivar, Kasalath, is very highly competent for *Agrobacterium*-mediated transformation compared to Nipponbare, suggesting that visual selection would be achieved more efficiently in Kasalath than in Nipponbare. We confirmed that the number of transformed cells emitting green fluorescence in *Agrobacterium* co-cultivated callus of Kasalath under no antibiotic pressure was >50-fold higher than the number obtained in Nipponbare. We succeeded in clonal propagation of GFP emitting cells at a frequency of 62.2–85% in Kasalath. Moreover, we showed that anthocyanin, that is an intrinsic pigmentation and thus might be more acceptable to consumers, can be used as an intrinsic marker in visual selection of rice. Thus, we propose that combination of the use of cultivar which has high transformation competency and visual selection system could enhance efficient production of transgenic rice expressing foreign gene product at higher level.

Key words: *Agrobacterium*, *indica* rice, transformation frequency, visible selection.

A suitable selection marker is essential to allow selection of successfully transformed cells from a large number of non-transformed cells. The use of antibiotic resistance markers such as hygromycin (hyg) phosphotransferase (hpt) has been a subject of public concern, due to the possible deleterious effects on other organisms in the ecosystem caused by the horizontal transfer of antibiotic-tolerant genes. Furthermore, in transformation experiments, antibiotics cannot be used for the selection of plant cells that are too sensitive to antibiotic pressure. Visual selection is a potentially useful alternative method of selection that could be used to produce transgenic plants without antibiotic selection. The application of visual selection to this system could be useful in the production of more widely acceptable transgenic crops. Of the visible markers currently available, fluorescent proteins such as green fluorescent protein (GFP) enable direct observation of transformed cells under living conditions without the need for treatment with exogenous substrates (Hraska et al. 2006; Stewart 2001). Moreover, GFP fluorescence can be an indicator of recombinant protein synthesis in transgenic plants as there is a positive correlation between GFP protein concentration and GFP fluorescence (Richards et al. 2003). Thus, visual selection without drugs can be applied not only for the production of lower risk genetic engineering for crop but also for the establishment of improved plant cell culture systems to produce recombinant proteins. However, it had been pointed out that the overall transformation efficiency using visual selection remains low due to the technical difficulties involved in clonal propagation of transformed cells following visual selection in rice, sugarcane and wheat (Elliott et al. 1999; Jordan 2000; Saika and Toki 2009; Vain et al. 1998).

To date, visual selection using a GFP marker without antibiotics in rice has been established in a transformation system using particle bombardment of immature embryos and *Agrobacterium*-mediated transformation of callus derived from mature seeds (see Supplemental Figure 1) (Saika and Toki 2009; Vain et al. 1998). In our previous report, we succeeded in achieving clonal propagation of transformed calli at a frequency of 9.4–20.5% in Nipponbare using visual selection with GFP

Abbreviations: DFR, Dihydroflavonol 4-reductase; GFP, Green fluorescent protein; Hyg, Hygromycin; hpt, Hygromycin phosphotransferase
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(Saika and Toki 2009). In the clonal propagation step in Nipponbare, cells emitting green fluorescence were not observed in ~50% transformed callus (10 of 24 primary calli) grown on medium without Hyg at 10–14 days after the onset of *Agrobacterium* elimination (Supplemental Table 1), suggesting that an improvement of transformation efficiency could increase clonal propagation efficiency when using visual selection in rice. In a recent report, we discovered that Kasalath, a model variety of *indica* rice, showed high competency for *Agrobacterium*-mediated transformation in our transformation system and independent transformation events in primary callus of Kasalath occurred successfully at ca. 10-fold higher frequency than in Nipponbare under Hyg pressure (Saika and Toki 2010). To verify whether more cells emitting green fluorescence were obtained in Kasalath than in Nipponbare under no antibiotic pressure, *Agrobacterium*-co-cultivated calli were grown on medium without Hyg for 14 days. Under no Hyg pressure, more transformed cells emitting green fluorescence were seen in Kasalath than in Nipponbare (Figure 1; Supplemental Figure 2A, B). The number of cell clusters emitting green fluorescence in *Agrobacterium*-infected callus derived from one mature seed of Kasalath was 47.3 ± 9.82 GFP expressing sectors/callus, i.e., >50-fold higher than in Nipponbare (0.88 ± 0.19) (Supplemental Table 1). Transformed cells emitting GFP were propagated successfully in both Nipponbare and Kasalath under conventional Hyg selection (Supplemental Figure 2C, D). This suggested that Kasalath might be more suitable for visual selection.

Using the visual selection system, we succeeded in achieving clonal propagation of transformed calli of Kasalath and regeneration of transformed plants. The efficiency of clonal propagation (the ratio of the number of clonal lines propagated successfully to the number of *Agrobacterium*-infected calli) obtained by visual selection in Kasalath was estimated at 62.2–85% (Table 1), compared to 9.4–20.5% achieved in Nipponbare (Saika and Toki 2009). We compared the details of the

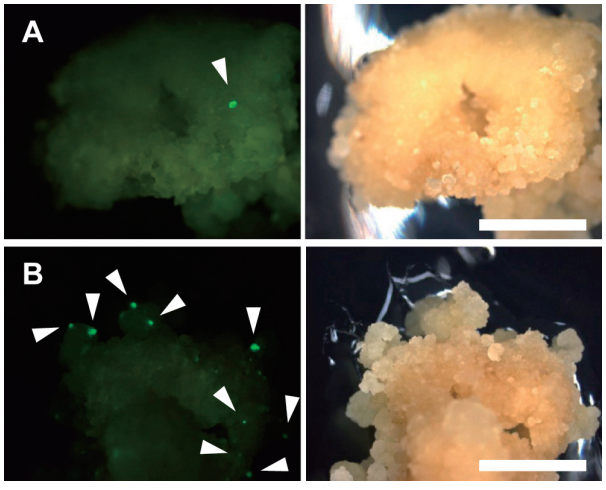

Figure 1. Transformed calli of Kasalath without Hyg selection. Representative transformed calli of Nipponbare and Kasalath without Hyg selection. Other examples are shown in Supplemental Figure 2A, B. Rice calli in Nipponbare and Kasalath were transformed with *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) harboring the binary vector shown in Figure 2A, and cultured without Hyg selection for 14 days. White arrowheads indicate GFP-emitting cell sectors. Panels A and B show transformed calli of Nipponbare and Kasalath, respectively. Left and right photos were taken under blue and white light, respectively. Bar=5 mm.

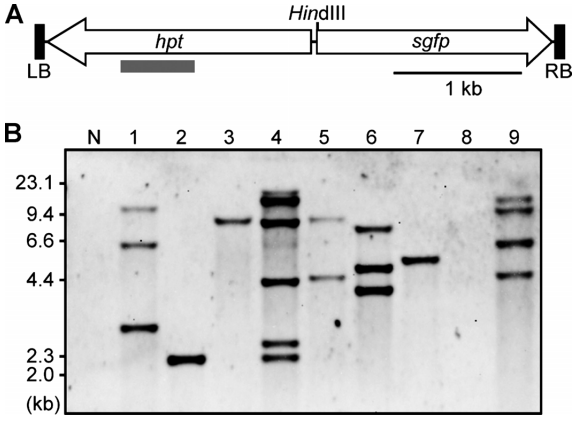

Figure 2. Southern blot analysis of regenerated Kasalath plants. (A) Vector construct used in this study. *sgfp* expression was under the control of the Cauliflower Mosaic Virus 35S (CaMV 35S) promoter and a duplicated nopaline synthase terminator (Niwa 2003; Niwa et al. 1999); *hpt* expression was directed by a duplicated CaMV 35S promoter and the 35S polyA signal. The gray bar indicates the region covered by the DIG-labeled *hpt* probe used for Southern blot analysis. LB, left border; RB, right border. (B) Southern blot analysis of *Hin*dIII-digested genomic DNA extracted from regenerated plants (T_0 generation) using the DIG-labeled probe shown in (A). Experimental procedures were performed following the method of Saika and Toki (2009). Lanes: NT, non-transformant; 1–9, plants from transgenic calli obtained by visual selection. GFP fluorescence was observed in every line analyzed in this study.

Table 1. Efficiency of visual selection in Kasalath

Experiment	No. of *Agrobacterium*-infected calli(A)	No. of lines successful in clonal propagation (B)[a]	No. of lines successfully regenerated (C)[a]	Visual selection efficiency (B/A, %)[b]	Regeneration efficiency (C/B, %)
Exp. 1	36	28	16	77.8	57.1
Exp. 2	20	17	10	85	58.8
Exp. 3	37	23	9	62.2	39.1

[a] Primary callus derived from one seed was counted as one line.
[b] This difference between Nipponbare and Kasalath was significant at $P<0.01$, as determined by *t*-test.

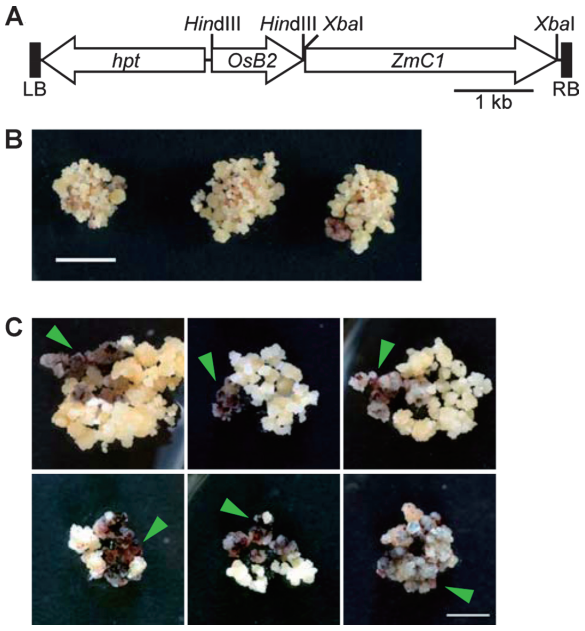

Figure 3. Visual selection using anthocyanin pigmentation. (A) Vector construct used in this study. *OsB2* and *ZmC1* expression was under the control of 35S promoter and nopaline synthase terminator; *hpt* expression was as described above. To construct pCAMBIA1390-B2C1 vector, *ZmC1* and *OsB2* expression cassettes were cloned into the binary vector pCAMBIA1390 using *Xba*I and *Hin*dIII, respectively. (B) Rice calli of Kasalath were transformed with *Agrobacterium* harboring the binary vector shown in (A), and cultured under Hyg selection for 25 days. Purple cell sectors were observed in Hyg-tolerant calli. Bar=1 cm. (C) Rice calli transformed with *Agrobacterium* harboring the binary vector shown above were cultured on N6D medium containing no Hyg for 47 days (visual selection was performed 3 times). Green arrowheads indicate representative purple cell sectors from which we successfully isolated anthocyanin accumulating cells. Bar=5 mm.

clonal propagation step between Nipponbare and Kasalath. At the time of the first transfer of cells emitting green fluorescence to fresh medium, at least one GFP positive cell cluster were observed in almost all calli of Kasalath (86.4–100.0%; average 94.6%) compared to 21.9–67.1% (average 40.2%) of the tested calli in Nipponbare (Supplemental Table 2). Moreover, we found that GFP-positive transformed cells could be propagated as efficiently as non-transformed cells in Kasalath (Supplemental Figure 1). These results further support our suggestion that it is easier to select transformed cells with GFP fluorescence in Kasalath than in Nipponbare.

Southern hybridization analysis was performed to evaluate the presence of the T-DNA in the T_0 generation of transgenic rice plants. On average, 2.5 T-DNA copies ($n=13$) were integrated in rice plants selected with GFP (Figure 2B). In our previous report, 2.4 copies of T-DNA, on average, were integrated in rice plants selected on Hyg in Kasalath (Saika and Toki 2010), suggesting that the integrated T-DNA copy number in regenerated plants selected visually is comparable to that in plants obtained by Hyg selection in Kasalath. In addition, on average, 2.3 T-DNA copies were integrated in rice plants of Nipponbare selected visually with GFP (Saika and Toki 2009), suggesting that the integrated T-DNA copy number in regenerated plants of Kasalath selected by visual selection is comparable to that of Nipponbare. An inverse correlation between T-DNA copy number and gene expression has been commonly reported in a wild-type *Arabidopsis* background where gene silencing is active, although a positive correlation is observed in a gene silencing mutant background (Luo and Chen 2007). However, it was shown that transgene expression levels varied among transformants containing a single copy T-DNA insertion (Luo and Chen 2007). Taken together, these findings indicate that visual selection enables efficient selection of transformed cells expressing high levels of GFP regardless of T-DNA copy number. In addition, the possibility exists that transformed cells in which transgenes are expressed at much higher level could be selected efficiently by the suppression of RNA silencing in Kasalath as well.

The stable inheritance of transgenes to the T_1 generation of transformed plants was evaluated by observation of GFP fluorescence. GFP fluorescence was observed in 32 of 44 T_1 seeds in line #2 shown in Figure 2B and 35 of 51 T_1 seeds in line #3, which fits a 3:1 ratio ($\chi^2=0.12$, $P=0.72$ in line #2 and $\chi^2=1.10$, $P=0.29$ in line #3). Thus, transgenes in transformed plants selected visually using GFP fluorescence are stably inherited to the T_1 generation in a Mendelian manner.

We checked whether visible markers other than fluorescent proteins could be applied successfully to our visual selection method. We focused on anthocyanin because it is an intrinsic pigment, and thus might be more acceptable to consumers than an exogenous marker when transgenic plants selected by visual selection are released on the market. Previous reports have shown that anthocyanin pigmentation in rice requires three types of dominant genes encoding two kinds of transcription factors [B1 or B2 (basic helix-loop-helix), and C1 (R2R3-Myb)] and the enzyme responsible for anthocyanin biosynthesis: dihydroflavonol 4-reductase (DFR) (Furukawa et al. 2007; Nagao and Takahashi 1963; Saitoh et al. 2004; Sakamoto et al. 2001). Moreover, anthocyanin pigmentation has been observed in rice calli transformed with a vector expressing these two kinds of transcription factor isolated from maize (Gandikota et al. 2001). DFR is known to be functional in Kasalath (Furukawa et al. 2007). We confirmed anthocyanin pigmentation in calli of Kasalath transformed with 35S::*OsB2* and 35S::*ZmC1* constructs grown on medium with Hyg (Figure 3B), confirming that anthocyanin pigmentation could be used as a visible marker in rice transformation. Indeed, we succeeded in clonal propagation of anthocyanin-accumulating calli by application of our visual selection system (Figure 3C).

Thus, we successfully improved the efficiency of visual selection by using the *indica* rice model cultivar Kasalath, which is highly susceptible to *Agrobacterium*-transformation, and demonstrated that higher transformation competency can improve visual selection efficiency in rice. We showed that the number of transformed cells emitting green fluorescence of Kasalath under no antibiotic pressure was >50-fold higher than the number obtained in Nipponbare (Table 1). In our previous report, regeneration efficiency in Kasalath was shown to be about half that in Nipponbare (Saika and Toki in press). Taken together, it is estimated that transformed plants of Kasalath are successfully obtained at a 25-fold higher frequency by visual selection than that of Nipponbare. The establishment of highly efficient visual selection will allow this technology to be applied not only to the production of risk-free transgenic crops but also to the successful transformation of plants recalcitrant to selection with antibiotics.

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