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Transgenic Note

Identification of hemizygous and homozygous transgenic rice plants in T_1 generation by DNA blot analysis

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Abstract Southern hybridization-based zygosity analysis was done in a transgenic rice plant (*Oryza sativa* L. cv Pusa Basmati 1) generated by *Agrobacterium*-mediated transformation with a rice chitinase (*chi*11) gene. A T_0 plant with two unlinked T-DNA insertions (A and A′), was chosen for the application of Southern hybridization analysis to study genetic separation of the two loci by segregation and to identify the homozygous and hemizygous plants in T_1- generation. The T_1 plants showed differences in band intensities that reflected the homozygous and hemizygous status of each of the two integration events. The predictions of zygosity of T_1 plants were confirmed by analyzing segregation in T_2 plants. Southern hybridization analysis is demonstrated as a simple and effective method to distinguish hemizygous and homozygous plants in the T_1 generation itself.

Key words: Genetic separation, T_1 generation, transgenic rice, zygosity analysis.

Southern hybridization analysis is crucial in the molecular characterization of transgenic plants. The number of T-DNA integration events is determined by junction fragment analysis (Hiei et al. 1994). Simple integration events with single, complete T-DNA copies, integration events with tandem dimers, dimers with invert repeats, truncated copies of T-DNA and long transfer events can be easily recognized by Southern hybridization analysis (De Neve et al. 1997; Kim et al. 2003; Sridevi et al. 2003). Co-transformation of two T-DNAs in the T_0 plants and their genetic separation in the T_1 generation were demonstrated by Southern hybridization analysis (Komari et al. 1996).

The homozygous and hemizygous transgenic plants should be differentiated for two important reasons. Hemizygous and homozygous plants may differ in their phenotype due to transgene dosage effect (Dai et al. 1999). Homozygous plants are true breeding and all their progeny will carry the transgene in the homozygous state. Usually, genetic analysis helps in the identification of homozygous and hemizygous transgenic plants. When single-copy transgenic (T_0) plants are selfed, the T_1 plants will have the following genotypes: homozygous for the presence of the transgene (1/4), homozygous for the absence of the transgene (1/4) and hemizygous for the transgene (2/4). Conventionally, the zygosity of the T_1 plants is determined by selfing them and by analyzing the presence of transgene in the T_2 seedlings on the basis of antibiotic or herbicide resistance. Segregation analysis is usually performed with the seeds of 6 to 9 T_1 plants that carry the transgene. A T_1 plant is considered homozygous if all its T_2 progeny carry the transgene. Identification of homozygous T_1 plants by molecular analysis saves one generation time.

Many molecular approaches have been used to determine zygosity of the transgene in the T_1 generation itself. Fluorescence *in situ* hybridization was used to detect homozygous barley plants harbouring *uid*A, *sgfp* and *bar* genes (Choi et al. 2002). Real-time PCR study using the standard curve-based absolute quantification method was used to identify homozygous and hemizygous soybean with *cryIAc* transgene and peanut with *hph* transgene (Schmidt and Parrot 2001). In the same work, the ‘comparative Ct (threshold cycle) method’ was used for the relative determination of copy number in soybean T_1 plants by comparing the amplification of the transgene (*cryIAc*) to that of an endogenous gene (lectin gene *Le1*) in a multiplexed PCR

Abbreviations: *bar*, bialaphos resistance gene; *chi*11, rice chitinase gene; *int-gus*, intron-containing β-glucuronidase gene; PPT, phosphinothricin.
This article can be found at http://www.jspcmb.jp/

reaction. The Ct values were found to be different for homozygous and hemizygous plants. The comparative Ct method was deployed for zygosity analysis of T_1 tomato plants harbouring tomato hexokinase gene in sense and antisense orientations (German et al. 2003). An endogenous, single copy vacuolar invertase gene was used as an internal control. The parent T_0 plant served as a calibrator. In the present study, zygosity of the transgenic rice plants was determined in the T_1 generation on the basis of comparison of band intensities of the transgene in Southern hybridization analysis.

A binary vector, pMKU-RF1 (Figure 1A) was constructed by subcloning a 3.1-kb *Hind*III fragment containing rice chitinase (*chi*11) (Huang et al. 1991) gene under maize ubiquitin promoter-intron into pCAMBIA3301 obtained from the Center of Application of Molecular Biology to International Agriculture (CAMBIA), Canberra, Australia. The vector has *bar* gene as a plant selection marker and β-glucuronidase gene with intron (*int-gus*) as a reporter. The binary vector was mobilized into *Agrobacterium tumefaciens* strain LBA4404 harbouring pSB1 (a plasmid that carries *vir*B, *vir*G and *vir*C from pTiBo542; Komari et al. 1996).

The scutellum-derived calli of rice (*Oryza sativa* L. cv Pusa Basmati 1) were transformed with *A. tumefaciens* strain LBA4404 (pSB1, pMKU-RF1) as described earlier (Sridevi et al. 2003). The transformed calli were selected using $8\,mg\,l^{-1}$ phosphinothricin (PPT, ammonium salt of glufosinate) and regenerated on $4\,mg\,l^{-1}$ PPT. Regenerated plants were established in a greenhouse.

Total DNA extracted (Rogers and Bendich 1998) from PPT-resistant plants was quantified in a DNA fluorometer (DyNA Quant 200) using Hoechst dye 33258 (Brunck et al. 1979). Five microgram aliquots of DNA were digested with *Hind*III, separated in a 0.8% agarose gel and transferred onto Zeta-probe nylon membrane (Bio-Rad, Hercules, CA) for Southern hybridization analysis. A 2.0-kb *int-gus* sequence was labeled with [α-^{32}P]dCTP and used as probe to detect right border junction fragments which are expected to be longer than 3.0-kb (Figure 1A). One T_0 plant, *chi-bar*6, that carried two junction fragments (designated as A and A′ in Figure 1B) was taken up for segregation analysis. The seeds of the selfed *chi-bar*6 were germinated in dark on half-strength Murashige and Skoog (MS) medium with 0.8% agar. The sprouted seeds were transferred to $^1/_2$ MS medium containing $5\,mg\,l^{-1}$ PPT and placed under light (16 h light/8 h dark photoperiod). The number of PPT^R and PPT^S T_1 seedlings was scored. The observed segregation ratio of 15 : 1 (data not shown) confirmed that the loci A and A' in the transgenic line *chi-bar*6 are unlinked.

The two-copy *chi-bar*6 line was selfed and the T_1 plants were analyzed for genetic separation of the two transgene loci A and A′ and to identify the homozygous

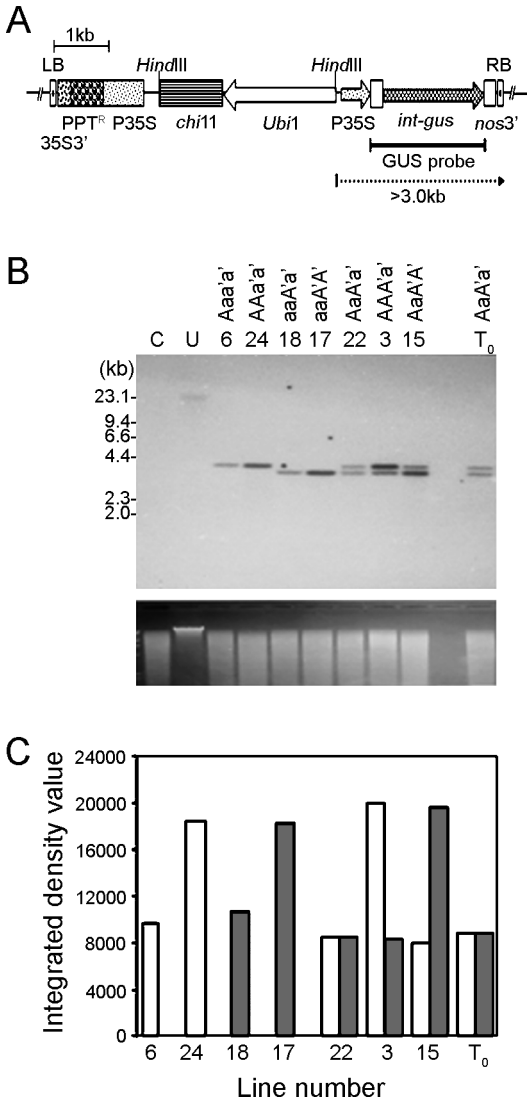

Figure 1. DNA blot analysis. (A) A map of pMKU-RF1 T-DNA region. The binary vector pMKU-RF1 carries *chi*11 gene under maize ubiquitin promoter-intron (*Ubi*1) in pCAMBIA3301. The T-DNA region also has *int-gus* as a reporter gene and bar (PPT^R) gene for plant selection. The probe region is indicated as a bold line and the junction fragment generated near RB is indicated in dotted lines. P35S-*Cauliflower mosaic virus* 35S promoter; 35S3′-*Cauliflower mosaic virus* 3′ region; *nos*3′-nopaline synthase 3′ region. (B) DNA blot analysis of T_1 plants of the T_0 line *chi-bar*6 using *int-gus* as probe. Genomic DNA (2.5 μg) from seven representative T_1 plants as well as the T_0 plant were digested with *Hind*III and separated in a 0.8% agarose gel. Lanes-C, DNA from control plant digested with *Hind*III; U, undigested DNA from T_1 plant 3. The numerals on the top represent the numbers assigned to the T_1 plants. The predicted genotype is marked on the top (e.g. AaA′a′ for plant 22). The sizes of λ-*Hind*III fragments are marked on the left. (upper panel) The top portion of the ethidium bromide-stained gel before blotting is shown to reflect the uniformity of DNA loaded in each of the lanes. (lower panel) (C) Analysis of signal intensity of autoradiogram. Each bar in the histogram represents the integrated density value of the junction fragment designated as A (unshaded) and A′ (shaded). The T_1 plant numbers are marked in the bottom.

T_1 plants for each locus. Total DNA from 27 PPT^R T_1 plants was quantified accurately in a DNA fluorometer and subjected to Southern hybridization analysis (data not shown). The expected genotypes are AAA′A′, AAA′a′, AaA′A′, AaA′a′, AA′a′a′, Aaa′a′, aaA′A′ and aaA′a′. A and A′ denote the presence of transgene copies and a and a′ denote their absence. A Southern hybridization analysis of seven chosen T_1 plant samples, corresponding to seven distinguishable genotypes on the basis of band intensities, is represented in Figure 1B. The *int-gus* sequence was used as probe.

The predicted genotypes of the plants analyzed in Figure 1B are, Aaa′a′ (6-6), AAa′a′(6-24), aaA′a′ (6-18), aaA′A′(6-17), AaA′a′(6-22), AAA′a′(6-3), and AaA′A′(6-15). The eighth genotype AAA′A′, homozygous for both integration events, was not observed in the analysis of 27 T_1 plants. The above interpretations were further strengthened by comparing the band intensities of A and A′ in T_1 plants to those in the T_0 plant in which both the loci are hemizygous (AaA′a′). The plant 6-24 is homozygous for the integration event A and the plant 6-17 is homozygous for the integration event A′. The ethidium bromide stained gel (Figure 1B) shows that equal amounts of DNA were analyzed from all plants. The autoradiogram corresponding to Figure 1B was scanned and the image was analyzed using Alpha-Ease™ software. The integrated density values corresponding to A and A′ bands are presented in Figure 1C. The hemizygous and homozygous status of the different T_1 plants became clear when the values of T_1 plants were compared to those of the T_0 plant, which served as a calibrator.

The prediction of zygosity of T_1 plants of *chi-bar*6, made on the basis of Southern hybridization analysis, was verified by performing segregation analysis in the T_2 generation. The T_1 plants were selfed and their progeny were scored for PPT^R and PPT^S. Southern hybridization data revealed that T_1 lines 6-24 (AA) and 6-17 (A′A′) were homozygous. Accordingly, all the T_2 plants of the T_1 lines 6-24 and 6-17 were PPT^R (Table 1). Thus, the homozygosity of the lines 6-24 and 6-17 is confirmed. In the case of T_1 lines 6-6 (Aa) and 6-18 (A′a′), the progeny segregated in a 3 : 1 ratio (hemizygous state) suggesting that the trait (PPT^R) is controlled by a single locus. The progeny of the T_1 plant 6-22 (AaA′a′) segregated in a 15 : 1 ratio suggesting that PPT^R is encoded in two loci, A and A′. Segregation analysis in the T_2 generation confirmed all the predictions made on zygosity in the T_1 generation on the basis of Southern hybridization analysis. Segregation analysis was not done for the plants 6-3 and 6-15 with complex genotypes.

In conventional analysis of transgenic plants, homozygous T_1 plants are identified by performing segregation analysis in the T_2 generation. Identification of a homozygous line in the T_1 generation itself saves one generation time of about four months in the case of rice. Real-time PCR assays involving TaqMan technology were found to be useful in zygosity analysis of plants. The standard curve method (Schmidt and Parrot 2001) and the comparative Ct method (Schmidt and Parrot 2001; German et al. 2003) were reported to be useful in zygosity analysis. German et al. (2003) found that the Ct values of the parent T_0 plant served as a calibrator for identifying homozygous and hemizygous T_1 plants. Although real-time PCR offers advantages such as (i) it is less time consuming and (ii) it permits simultaneous analysis of a large number of T_1 plants, Bubner et al. (2004) highlighted a limitation in the application of real-time PCR in zygosity analysis. They pointed out that the detection limit of real-time PCR analysis is not sensitive enough to measure two-fold differences, which is needed to distinguish between hemizygous and homozygous plants. Real-time PCR-based zygosity analysis was not feasible for transgenic mice harbourimg multiple, unlinked transgenes (Shitara et al. 2004).

Table 1. Segregation analysis of T_2 plants derived from representative hemizygous and homozygous T_1 plants of the T_0 line *chi-bar*6.

Selfed T_1 plant number	Predicted genotype	Number of T^2 seedlings[a]			Expected ratio	χ^2 value
		Total	PPT^R	PPT^S		
24	AAa′a′	39	39	0	4 : 0	0
17	aaA′A′	37	37	0	4 : 0	0
6	Aaa′a′	36	27	9	3 : 1	0
18	aaA′a′	31	23	8	3 : 1	0.096
22	AaA′a′	64	58	6	15 : 1	1.067

[a] Scoring for the presence of the transgene was done on the basis of germination on PPT-containing medium (5 mg l^{-1}). The two integration events are designated as A/a and A′/a′.

The present study on the transgenic rice line *chi-bar*6 clearly demonstrates the combined advantage of Southern hybridization in genetic separation of two unlinked integration events and in identifying the homozygous line in the T_1 generation itself. Though we report here the results of only one line, this approach was successful for routine zygosity analysis in more than 10 transgenic rice lines with single or two copy insertions (data not shown). Southern hybridization analysis offers many advantages in zygosity analysis: (i) it is routinely done to determine transgene copy numbers in the T_0 generation and has to be simply extended to the T_1 generation for zygosity analysis, (ii) it gives information on zygosity and also on the fingerprint of the integration event, (iii) the genetic separation of two unlinked integration events and zygosity can be studied simultaneously, which is not readily feasible through real-time PCR (Shitara et al. 2004), and (iv) it can be performed under simple laboratory conditions and is equally informative as real-time PCR for zygosity

analysis.

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