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Regeneration of Transgenic Rice with Bacterial *ipt* Gene Driven by Senescence Specific (*SAG12*) Promoter by Particle Bombardment

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Abstrak: Pokok padi transgenik telah dijanakan menggunakan pembedilan partikel untuk memperkenalkan gen biosintesis sitokinin *Agrobacterium* yang dipacu oleh promoter spesifik kesenesensan Arabidopsis (*Arabidopsis thaliana*) iaitu *SAG12,* yang berfungsi untuk melengahkan kesenesensan daun. Dengan menggunakan dua plasmid, kalus embriogeni berumur satu minggu yang diambil dari padi Japonica (*Oryza sativa*) varieti Chin Guang matang telah ditransformasi. Gen penanda memilih hygromycin phosphotransferase (*hph*) dan gen *reporter* B-ß-glucuronidase (*uidA*) yang kedua-duanya dikawal oleh promoter *cauliflower mosaic virus* (CaMV) 35S, telah diletakkan pada vektor *co-integrate* manakala gen biosintesis sitokinin, isopentenyl transferase (*ipt*) yang dipacu oleh promoter *SAG12* telah dibekalkan dalam plasmid yang lain. Tiga puluh dua pokok padi transgenik telah dijana; 27 pokok telah dipilih secara rawak untuk cerakin histokimia ß-glucuronidase (GUS), PCR dan analisis sap *Southern*. Frekuensi *co-integration* sebanyak 88% dan 69% telah diperolehi untuk dua gen terangkai (*uidA* and *hph*) dan dua gen tidak terangkai (*hph* and *ipt* gene) masing-masing dalam pokok R_0. Analisis sap *Southern* telah mensahkan keputusan cerakin histokimia GUS dan amplifikasi PCR. Satu corak integrasi yang kompleks untuk semua transgen termasuk integrasi salinan berbilang telah diperhatikan.

Kata kunci: *Co-transformation*, Pembedilan Partikel, Gen *ipt*, Promoter *SAG12*, Padi

Abstract: Transgenic rice plants were generated using particle bombardment to introduce the *Agrobacterium* cytokinin biosynthesis gene driven by Arabidopsis (*Arabidopsis thaliana*) senescence specific promoter (*SAG12*) for delaying leaf senescence. Using two plasmids we co-transformed one week old embryogenic calli derived from mature Japonica rice (*Oryza sativa*) variety Chin Guang. The selectable marker hygromycin phosphotransferase (*hph*) gene and the reporter gene B-ß-glucuronidase (*uidA*), both under the control of cauliflower mosaic virus (CaMV) 35S promoter were placed on the same co-integrate vector whereas the cytokinin biosynthesis gene, isopentenyl transferase (*ipt*) driven by the *SAG12* promoter is supplied in another plasmid. A total of 32 transgenic rice plants were regenerated of which 27 plants were randomly selected for histochemical ß-glucuronidase (GUS) assay, PCR and Southern blot analysis. Co-integration frequencies of 88% and 69% were obtained for two linked genes (*uidA* and *hph*) and two unlinked genes (*hph* and *ipt* gene) respectively in R_0 plants. Southern blot analysis confirmed the results of histochemical GUS assay and PCR amplifications. A complex integration pattern for all the transgenes including the multiple copies integration was predominantly observed.

Keywords: Co-transformation, Particle Bombardment, *ipt* Gene, *SAG12* Promoter, Rice

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INTRODUCTION

Rice is one of the most important cereal crops and is the major food source for three billion people comprising more than a third of the world's population. More than 90% of the world's rice is grown and consumed in the Asia Pacific region where 60% of the earth's population live (IRRI 2010). Global rice production for the year 2012–2013 is projected at a record of 480.5 million tons [Food and Agriculture Organization (FAO 2012)] which must be increased to 850 million tons by 2025 to meet the demand. This is a supreme challenge which calls for a multi-pronged approach to develop rice varieties with higher yield potential and yield stability. Genetic engineering of rice with agronomical important genes could be one of the approaches.

Besides pests, disease and several abiotic stresses, another burning problem that limits rice productivity is poor grain filling. Grain yield is mainly associated with two physiological processes: photosynthesis which produces carbohydrate and partitioning which determines the carbohydrate storage in developing grain. Two strategies could be adopted to address the problem of grain filling. The first is through modifying the endogenous plant hormone (auxins and cytokinins) level during grain development and the second is through delaying leaf senescence by introducing cytokinin biosynthesis gene thus prolonging the time during which leaves can make carbohydrate by photosynthesis and supply the carbohydrates to the developing grain.

In rice, senescence of the top three leaves (flag leaves) between flowering and grain development appears to be critical in determining the grain filling. Although leaf senescence is thought to be an evolutionary adaptation to recycle nutrient, the ability to control senescence has significant impact on yields in an agriculture setting. For example in some maize hybrids, delay of leaf senescence is associated with increase in yields (Duvick 1984). Cytokinin class of plant hormones are known to delay senescence in plants. However exogenous application of cytokinin is imprecise and could have undesirable side effects. The auto regulated production of cytokinin by senescence specific promoter using a transgenic technology could be an alternative approach for delaying leaf senescence (Gan & Amasino 1995). In recent years several senescence associated genes were identified and transgenic approach has been deployed in many plant species which demonstrated that endogenously produced cytokinin can regulate senescence and provide a system to specifically manipulate the senescence program (Quirino *et al*. 2000; McCabe *et al*. 2001; Robson *et al.* 2004; Swartzberg *et al*. 2006; Sykorova *et al*. 2008). In this paper we report the genetic transformation of rice with bacterial isopentenyl transferase (*ipt*) gene (the enzyme that catalyses the rate limiting step in cytokinin biosynthesis) driven by a senescence specific (*SAG12*) promoter. The integration of transgene is confirmed through PCR genomic blot hybridisation.

MATERIALS AND METHODS

Plasmid Construction

Plasmid pSG516 contains *Agrobacterium tumefaciens ipt* gene fused to *Arabidopsis thaliana SAG12* promoter and nos terminator (Gan & Amasino 1995; Fig. 1). Plasmid pWRG1515 contains the B-ß-glucuronidase (*uidA*) reporter and hygromycin phosphotransferase (*hph*) selectable marker gene both controlled by the cauliflower mosaic virus (CaMV) 35S promoter (Christou & Ford 1995).

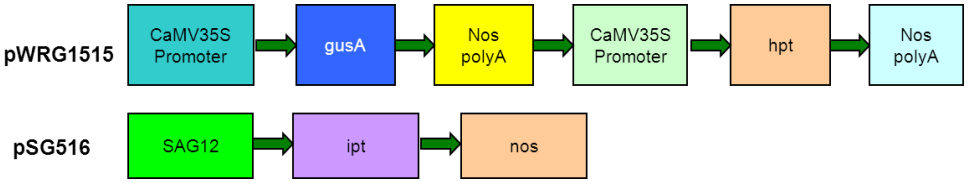

Figure 1: Schematic representation of the plasmids (pWRG1515 and pSG516) used in transformation experiments.

Explants and Transformation Procedure

Sterilised mature seeds of Japonica rice *Oryza sativa* cv. Chin Guang were cultured on the callus induction medium containing Linsmaier & Skoog (LS) medium (Linsmaier & Skoog 1965) supplemented with 2mg/l 2,4-D, 30g/l maltose solidified with 2.5 g/l phytagel ^{TM} (Sigma), (pH 5.8) at 28°C in dark. After 3 weeks, compact embryogenic calli were selected and calli were bombarded using a Biolistic PDS-1000/He (Bio-Rad, UK Ltd.) particle acceleration device under a chamber pressure of 27 mm Hg at a distance of 13 cm below the microprojectile stopping plate, with a helium pressure of 1100 psi as per the protocol described earlier (Ghosh Biswas *et al*. 1998). Two days after bombardment, calli were transferred to fresh callus induction medium containing 40 mg/l hygromycin B (Sigma-Aldrich, UK). In the selection medium, freshly developed calli were selected at 3 weeks interval and changed to new selection medium with 50 mg/l hygromycin B. After two rounds of selection, surviving calli were transferred to LS basal medium containing 3mg/l BAP, 30 g/l maltose, 40 mg/l hygromycin and 6.0g/l phytagel (pH 5.8). Transgenic plant regeneration and green house establishment was carried out following the protocol of Zhang *et al*. (1997).

Histo-chemical GUS Assay

ß-glucuronidase (GUS) assay was carried out according to the procedure described by Jefferson (1987). Tissue segments were immersed overnight in assay buffer containing 20% methanol (Merck, UK), 50 mM sodium phosphate buffer (pH 7), 500 µM potassium ferrocyanide, 500 µM potassium ferricyanide, 5 mM EDTA, 0.5% triton X-100 and 20 mM X-Glu (all from Sigma-Aldrich, UK). Following incubation, chlorophyll was removed in 70% ethanol (Fisher Scientific, Loughborough, UK) for visualisation of coloured products.

PCR and Southern Blot Analysis

Genomic DNA from the leaves of the primary transformants was isolated by the method of Michaels *et al.* (1994). The PCR reaction was carried out in a 50 µl volume containing 1 µl of template DNA, 1X Taq buffer, 200 µM dNTPs, 0.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 9.0), 0.1% v/v triton x and 2 units of *Taq* DNA polymerase. The *uidA* gene fragment was amplified using 5'TAGAAACCCCAACCCGTGAAATC3' and 5'CGACCAAAGCCAGTAAAGTAGAA3' primers, the *hph* gene with 5'CGCATAACAGCGGTCATTGACTGGAGC3' and 5'GCTGGGGCGTCGGTTTCCACTATCGG3' primers and, the *ipt* gene fragment was amplified using 5'CGTCTAATTTTCGGTTCAAC3' and 5'AGGGAATTTCTGTTCTTGTCG3' primers. Reactions were subjected to 35 cycles of 0.5 min at 95°C, 0.5 min 60°C and 1 min at 72°C with final extension at 72°C for 10 min. PCR products were separated in 1% agarose gels and were visualised with ethidium bromide.

Southern blot analysis was performed to confirm the stable integration of *ipt*, *hph* and *uidA* genes into the transgenic rice. DNA samples (5 µg) were digested with restriction endonuclease *Pst*I and or *Kpn*I and then fractioned on 0.8% agarose gels and transferred to a Hybond – N membrane (Amersham Pharmacia, Bukinghamshire, UK) according to manufacture's instructions. The *uidA* probe and *hph* probe were prepared from the plasmid pWRG1515. The 700 bp *ipt* probe was prepared from pSAG516 by restriction digestion with *NcoI*/*EcoRI* enzymes. The probes were labelled with α-^{32}P dCTP using rediprime labelling kit (Amersham Pharmacia, Bukinghamshire, UK). Membranes were washed twice at room temperature in 2X saline-sodium phosphate-EDTA (SSPE)/1% sodium dodecyle sulphate (SDS) for 10 min and at 65°C in 1X and 0.1X SSPE/0.1% SDS for 15 min each time and then autoradiographed using Amersham Hyper film.

RESULTS AND DISCUSSION

When mature seeds were incubated on callus induction medium containing maltose as a carbon source, more than 90% of seeds produced embryogenic calli. Compact, yellowish and granular embryogenic calli were selected for bombardment with gold particles coated with two separate plasmids, one carrying the *ipt* gene and the other carrying the *hph* selectable marker and *uidA* reporter genes. Two days after bombardment, bombarded calli were tested for GUS which revealed high frequency of transient GUS expression. After selection on 40 mg/l hygromycin B for 2 weeks, actively growing small cell clusters appeared on the surface of bombarded tissue. These tiny embryogenic cell clusters were assayed for GUS activity and several of these newly formed cell clusters stained intensely blue, indicating the stable transformation of rice. These fast growing cell clusters grow vigorously in hygromycin medium (50 mg/l) and when transferred to the regeneration medium they produced shoots.

Thirty two putative transformed plants were regenerated within three months from two separate experiments and transplanted in containment glass

house. Twenty seven putative transformants were selected for histochemical GUS assay of which 22 plants were tested GUS positive and 5 were GUS negative. The intensity of GUS staining varied considerably among the transformants and subsequent molecular analysis could not establish any relationship between the GUS intensity and transgene copy number. All the 27 plants were subjected to PCR analysis for *uidA*, *hph* and *ipt* transgenes. Out of the 27 transformants analysed, DNA of 26 transformants was amplified with *hph*, 23 with *uidA* and 18 with *ipt* transgenes. It was observed that the plants which tested GUS positive in histochemical GUS assay amplified both *gus* and *hph* transgenes. However, all the PCR GUS positive plants did not amplify *ipt* transgene whereas, all the PCR *ipt* positive plants amplified both *uidA* and *hph* transgenes (Fig. 2). This could be explained by the fact that both *hph* and *ipt* transgenes are in two different plasmids and since hygromycin B was used as a selective agent, it favoured the selection of hygromycin resistant callus lines and by the process, callus lines which have the *ipt* gene but not the *hph* gene might have got eliminated.

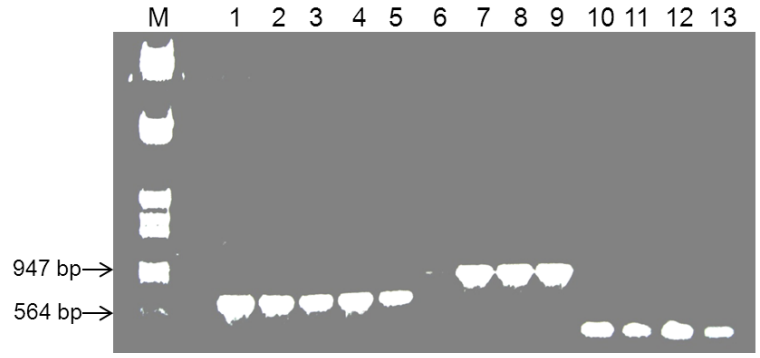

Figure 2: PCR analysis of transformed plants: M, λ DNA Hind III + EcoRI digested molecular wt. marker; lane 1, pSG516 plasmid amplified with *ipt* specific primers; lanes 2–5, *ipt* specific PCR amplification of transformed plants; lane 6, non transformed plant; lanes 7–9, GUS specific PCR amplification; lanes 10–13 *hpt* specific PCR amplification.

All the 18 PCR *ipt* positive transformants were selected for detail investigation for the integration of *ipt*, *hph* and *uidA* genes by Southern blot analysis. Results of Southern blot analysis of 10 transgenic plants are shown (Fig. 3). All the PCR *ipt* positive plants were confirmed to have integrated *ipt* transgene. When undigested transgenic genomic DNA was hybridised with the *ipt* probe, the hybridisation signal was observed only in the high-molecular weight region [Fig. 3(a)], indicating the integration of the transgene in rice. No hybridisation signal was detected in control non-transformed plants. DNA from PCR *ipt*-transgenic plants were digested with *pst*I restriction endonuclease to release the 3.18 kb fragment containing the *ipt* cassette with *SAG12* promoter and nos terminator region. Southern blots of these DNA hybridised with *ipt* probe revealed the presence of 3.8 kb fragment in 5 out of 9 transgenic plants indicating the presence of at least one intact copy of the coding sequence in

these plants. In addition to 3.8 kb fragment all the transgenic lines except one [Fig. 3(a); lane 7] contain several larger or smaller fragments suggesting that deletions, rearrangements and/or methylation of *pst*I site have occurred. In plant 7 [Fig. 3(b)] a single hybridisation band of less than 3.8 kb is observed.

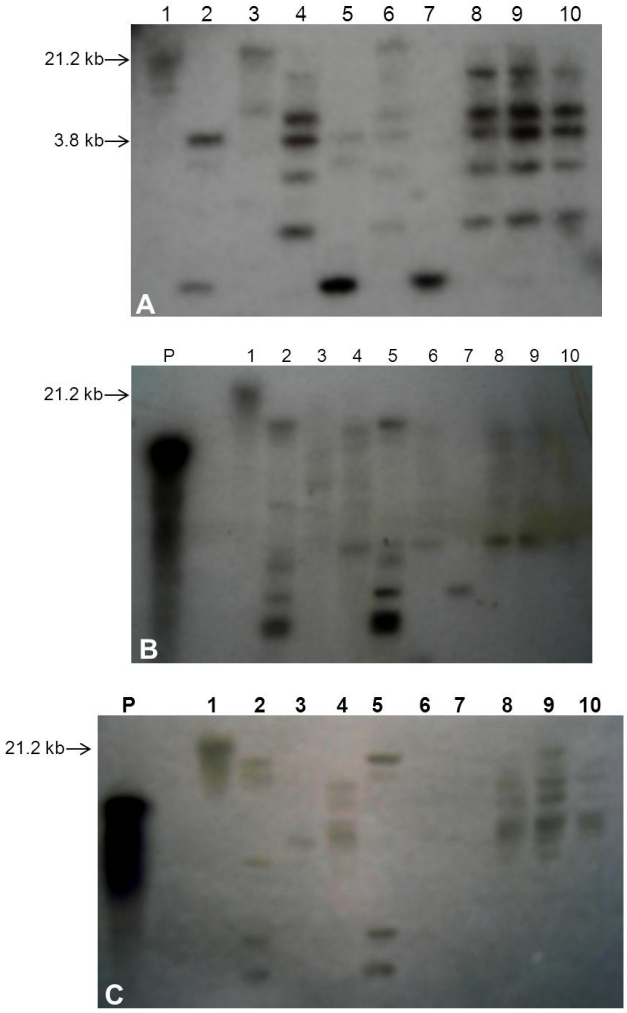

Figure 3: Southern blot analysis of transformed plants: (A) Southern analysis for the *ipt* gene in 10 plants randomly selected from different transformation events – lane 1, transgenic plant undigested; lane 2–10, transgenic plants digested with *pst*I; (B) Southern analysis for the *hph* gene in 10 plants randomly selected from different transformation events – P, plasmid pWRG1515; lane 1, transgenic plant undigested; lane 2–10, transgenic plants digested with *kpn*I; (C) Southern analysis for the *uidA* gene in 10 plants randomly selected from different transformation events – P, plasmid pWRG1515; lane 1, transgenic plant undigested; lane 2–10, transgenic plants digested with *kpn*I.

The transgene integration pattern of the *ipt* gene was complex as revealed from the Southern blot analysis. When the DNA of transgenic plants were digested with *sph*I (data not shown) which has a single site in the plasmid, 5.9 kb fragment (the size of the entire plasmid) was obtained in many transgenic plants. This fragment is most probably generated from multiple plasmid copies inserted as head to tail concatamers. Larger or smaller hybridising bands constitute plasmid plant junction DNA which is of different sizes and therefore represent either single copy integration events, the ends of concatamers or rearranged molecules within concatamers. Similar integration pattern of transgenes was observed in rice following bombardment with multiple plasmids (Tang *et al*. 1999).

The integration pattern of *uidA* and *hph* transgenes in rice genome was verified using the DNA from the transgenic lines by digesting with *kpn*I for which pWRG1515 has a unique restriction site. The filter was first hybridised with *hph* probe, stripped and latter used with the *uidA* probe. When undigested DNA from the transgenic plants were hybridised with the *hph* and *uidA* probes, hybridisation signal was observed only in the high molecular weight region [Fig. 3 (b) and (c)] indicating the integration of these two transgenes in rice genome. In both the cases, hybridising bands varying from 1–6 of different sizes were observed. A 7.0 kb fragment (size of the plasmid pWRG1515) was found to be present in at least four plants [Fig. 3. (b) and (c); lanes 4, 8, 9 and 10] which could be due to multiple insertions of plasmid copies as head to tail concatamers. In all the transgenic lines except at lane 7 [Fig. 3 (b) and (c)] multiple hybridising bands were observed which represent either independent single copy integration events or rearranged molecules within concatamers or concatamer ends. Such integration patterns have been observed in different transgenic plant species produced via variety of transformation methods (Xu & Li 1994; Rashid *et al*. 1996; Mishra *et al*. 2010). In the transgenic lines at lane 6 and 7, hybridisation bands were obtained using *hph* probe [Fig. 3(b)] but no band was observed using *uidA* probe [Fig. 3(c)]. This is probably due to the insertion of a truncated copy of the plasmid incorporating the *hph* coding sequence but not the *uidA* gene. Non uniform incorporation of the transgenes has been observed in many transformants obtained through particle bombardment (Wan & Lemaux 1994; Tang *et al.* 1999; Wang *et al*. 2006). In biolistic bombardment, transgenes were delivered to plant cells by physical means which involves precipitation of DNA on to gold particles after vortexing and delivery of the DNA coated particles to plant cells using high pressure and velocity. During these processes, the DNA might be sheared, and this may be one of the reasons for deletion and separate integration of the transgenes. However, detail molecular analysis need to be carried out to confirm the integrity of the inserted plasmids and their expression in transgenic rice plants.

In the present experiment, rice calli were bombarded with two different plasmids and this has allowed us to assess the co-integration of both linked and unlinked genes. The co-integration frequency of linked genes (*uidA* and *hph*) in our experiment was 88% which is lower than the 100% frequency reported in maize and rice (Klein *et al*. 1989; Tang *et al*. 1999) and wheat (Vasil *et al*. 1992) but higher than the 84% frequency obtained in barley (Wan & Lemaux 1994).

The co-integration frequency of unlinked genes in our experiment was 69% which is slightly lower than the 72% obtained in rice (Wakita *et al*. 1998) but higher than the 63% of black Mexican sweet maize (Spencer *et al*. 1990) transformants. The frequency of co-integration appears to vary in different species and also when different plasmids were used. The co-integration frequency of linked and unlinked genes can also vary between different experiments using same plasmids and also using similar experimental conditions. The co-transformation approach has the potential application in obtaining marker free transgenic plants (Wang *et al*. 2006). If the co-transformed genes are integrated in two different loci and not genetically linked, it could be feasible to obtain plants containing only one of the transgene via recombination. However, physical and genetic linkage between integration sites needs to be studied before addressing this approach.

In conclusion, we have produced transgenic rice plants with bacterial *ipt* gene (the enzyme that catalyses the rate limiting step in cytokinin biosynthesis) driven by senescence specific promoter with an objective to delay leaf senescence. The integration of transgene was confirmed through PCR and Southern blot analysis which revealed multiple copy insertion and complex integration pattern. This analysis will facilitate to tag the individual plants for further biochemical analysis especially the expression analysis of *ipt* transgenes as well as help us to study the inheritance pattern of transgenes in the progenies. This will probably allow us to gain insight and precisely manipulate the complex phenomenon of leaf senescence in rice.

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