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CONSTRUCTION OF EUKARYOTIC PLANT EXPRESSION VECTOR WITH THE SAG12 PROMOTER AND THE LEAF SENESCENCE–RELATED IPT GENE AND ITS GENETIC TRANSFORMATION IN RICE

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Abstract

A pair of primers was designed according to the sequences published by GenBank to amplify the isopentenyl transferase gene (ipt gene) of the plasmid in *Agrobacterium tumefaciens* (strain C58). The 5′-untranslated sequence of the senescence-associated *SAG12* gene of Arabidopsis was isolated by the polymerase chain reaction (PCR). In addition, we constructed the plant-expression vectors (pBI121-ipt and pCAMBIA1301-SAG12-ipt), which were regulated by the SAG12 promoter and the ipt gene. The plant-expression vector was used to transform the embryos of rice cultivar Zhonghua 16 with the help of the *A. tumefaciens* system. Plantlets were regenerated in vitro by selection on medium containing hygromycin. PCR amplification showed that the target gene was successfully integrated into the transformed plants.

Introduction

Plant hormones regulate plant growth and the production of physiological substances, such as hormones, in micro-quantities. Cytokinins (CTKs), a type of active plant hormones, are involved in the entire developmental process of plants. CTKs have for long attracted the attention of the research world for their role in the aging of plants, which delays plant senescence directly by promoting nutrient transport (Khodakovskaya *et al*., 2005; Ye *et al*., 2006), facilitating the synthesis of proteins and nucleic acids (Guivarc'h *et al*., 2002) and inhibiting ethylene production and other pathways (McCabe *et al*., 2001).

Molecular studies showed that CTK affected plant resistance and aging through the regulation of gene expression. Isopentenyl transferase is the rate-limiting enzyme in the catalytic synthesis of CTK, which is encoded by the isopentenyl transferase gene (ipt gene) of *Agrobacterium tumefaciens* (Smigocki *et al*., 2002). The senescence-associated gene *SAG12* gene encoding cysteine protease was cloned from Arabidopsis, which possessed highly specific expression during aging and could respond rapidly to external factors that accelerated the aging process (Guo *et al*., 2010; Pasquali *et al*., 2009; Sykorova *et al*., 2008). Therefore, an anti-aging self-adjusting system could be built by the ligation of the SAG12 promoter and the ipt coding region, which works as follows (Bai *et al*., 2009): When the leaves start aging, the SAG12 promoter is activated and the ipt gene begins to express itself, leading to an increase of the CTK concentration. Consequently, aging is suppressed. When the CTK concentrations reach a certain level, the symptoms of aging disappear, the SAG12 promoter is no longer active, nor is the ipt gene expressed; thus, the CTK concentration is controlled, thereby preventing an excessive negative impact of the plant hormones (Ma and Liu, 2009).

The ipt gene and the SAG12 promoter cloned in this study were used to construct the chimeric gene-expression vector, and the chimeric gene was introduced into the Japonica Zhonghua 16 genome using the *Agrobacterium*-mediated method. The transgenic lines with the chimeric gene obtained initially were identified by the polymerase chain reaction (PCR), which laid the foundation for investigating the influence of the chimeric gene expression on both leaf senescence of rice and rice yield, further improving the use of the chimeric gene to include applications aimed at anti-aging procedures in rice and other crops.

Materials and Methods

A.tumefaciens, C58 strain, was supplied by the Institute of Microbiology, Chinese Academy of Sciences; Arabidopsis (*Arabidopsis thaliana*), ecotype Columbia, was gifted by Pro. Chen Daqing of the Yangtze University; *Oryza sativa* Zhonghua 16, *Escherichia coli* DH5-α, the plasmid pCAMBIA1301 and *A. tumefaciens* EHA105 were procured from the preserved specimens in our laboratory. The pMD18-T vector and the enzymes, including restriction enzymes, *Taq* polymerase and ligase, were purchased from Takara Company.

Amplification, cloning and sequencing of the ipt gene: According to the sequence of the ipt gene of *A.tumefaciens* C58 published by the ***National Center for Biotechnology Information*** (AE009419), a pair of primers was designed, with the forward primer P1 (5′-tcagccatggatggatctgcgtctaattttcggtcc-3′) containing the start codon ATG and the *Nco*-I restriction site (underlined) and the reverse primer P2 (5′-gggtaccctaatacattccgaatggatgaccttcg-3′) containing the terminator and the *Bst*E-II restriction site (underlined). The Ti plasmid extracted from *Agrobacterium* C58 was used as the template; PCR amplification was carried out according to the following procedure: initial denaturation at 94°C for 5 min; 94°C for 30 s, 57°C for 30 s and 72°C for 1 min for 30 cycles; then, a final elongation at 72°C for 10 min. The PCR product was purified using a gel recovery kit (Omega), subsequently cloned into the pMD18-T vector and further sequenced.

Amplification, cloning and sequencing of P_{SAG12}: Total DNA was extracted using the cetyl trimethylammonium bromide (CTAB) method (Khodakovskaya *et al*., 2005). According to the SAG12 promoter sequence (ATU37336) reported in the GenBank, a pair of primers was designed. The upstream primer P3 (5′-cgcggatccagacactcgta ttagttggtactttggtag-3′) contains the *Bam*H-I restriction site (underlined), the downstream primer P4 (5′-catgccatggtgctcttaatttgattacttcagaaggg-3′).contains the *Nco*-I restriction site (underlined). The PCR reaction program was as follows: initial denaturation at 94°C for 5 min; 94°C for 1 min, 57°C for 30s and 72°C for 2 min for 30 cycles; then, a final elongation at 72°C for 10 min. The PCR product was purified, cloned into pMD18-T vector and sequenced.

Construction of plant-expression vector pBI121-ipt: Recombinant pMD18-ipt and pCAMBIA1301 were digested With *Nco*-I and *Bst*E-II, and then recovered prior to ligation. The ligation temperature was 16 °C, and the reaction system contained 2.5 µL of 10× T4 ligase buffer, 1 µL of T4 DNA ligase (5U/µL), 11.5 µL of the ipt gene and 10 µL of pCAMBIA1301 large fragments. The products were used for the transformation of DH5-α. A single colony was picked, followed by amplification using PCR and subsequent restriction-enzyme digestion.

Construction of pCAMBIA1301-SAG12-ipt plant-expression vector: pMD18-SAG12 and pCAMBIA1301-ipt were both digested with *Bam*H-I and *Nco*-I. The pCAMBIA1301-ipt large fragment and the SAG12 promoter were recycled before the ligation reaction. The fragments were incubated first at 16°C overnight and transferred to 4°C for several hours; the reaction mix contained the following: 2.5µL of 10× T4 ligase buffer, 1.5µL of T4 DNA ligase (5U/µL), 7µL of the SAG12 promoter gene, 13.5µL of the pCAMBIA1301 large fragment. The product was transformed into DH5-α, and a single colony was picked out before amplification using PCR and restriction-enzyme digestion.

***Agrobacterium*-mediated genetic transformation of rice and regeneration of transgenic plants:** The transformation was carried out according to the procedures in previous references (Khodakovskaya *et al*., 2006; Mei *et al*., 2011; Ye *et al*., 2006) with slight modifications: the shelled seeds were soaked in 70% ethanol for 5 min, sterilised in 0.1% mercuric chloride solution for 20 min, washed 4–5 times with sterile water and then inoculated into N6 induction medium. The callus culture was induced at 25 °C in the dark and sub-cultured once every 2–3 weeks. The loose callus, sub-cultured 2–3 times, was pre-cultured for 4–6 days to form a total culture. A single colony of *Agrobacterium* EHA105/pCAMBIA1301-SAG12-ipt was inoculated into yeast extract broth containing 50 mg/L of kanamycin and 25 mg/L of rifampicin, cultured to OD_{600} = 0.5–0.6, centrifuged, re-suspended in *Murashige and Skoog (*MS) medium and adjusted to OD_{600} = 0.5 (Shah *et al*., 2011a). Callus culture was pre-soaked in the bacterial suspension for more than 20 min, shaken incessantly, filtered through sterile paper to remove the excess bacteria and added to excess of N6-As medium and cultured at 24°C for three days in the dark. The co-cultured callus was first washed with sterile water 4–5 times, soaked in sterile water containing 500 mg/L cefalexin for 30 min, dried on sterile filter paper covered with three layers of the plate for 2 days. A batch of callus was taken in the selection medium N6S1 (N6 + hygromycin 25 mg/L) and transferred to N6S2 medium (N6 + hygromycin 50 mg/L) after two weeks. After 15–20 days, the anti-calli were transferred to the 3-layer sterile filter paper to dry for 1 day, then transferred to pre-differentiation medium [MS medium + Kinetin (KT) 0.5 mg/L + naphthalene acetic acid (NAA) 0.25 mg/L + 6-Benzylaminopurine (6-BA) 2 mg/L] in the dark and cultured for 7 days, then transferred to differentiation medium [MS medium + KT 2 mg/L + NAA 0.5 mg/L + IAA 0.25 mg/L] in the light, and grown to a length of 1–2cm; from here, it was transferred to the shooting medium (MS medium + KT 0.5 mg/L + NAA 0.5 mg/L + IAA 0.5 mg/L) and cultured for 3–4 weeks in the light. When the roots of the rice seedlings reached 15 cm in length, they were transplanted.

Molecular detection of resistant plants: A small amount of rice total DNA was extracted from the leaves using the CTAB method (Khodakovskaya *et al*., 2005). PCR was carried out with the primers P1 and P2 of the ipt gene, the primers P3 and P4 of the SAG12 promoter and the upstream and downstream primers of the SAG12 promoter for both the transgenic and control samples at the same time. The PCR products were detected on a 0.8% agarose gel.

Results

Amplification, cloning and sequencing of ipt gene: A 723-bp specific band was amplified using the Ti-plasmid of *Agrobacterium* C58 strain as the template, consisting of the expected fragment (Fig. 1). The fragment was recovered, purified and inserted into the pMD18-T vector to obtain the recombinant plasmid pMD18-ipt. The recombinant plasmid was digested with *Nco*-I and *Bst*E-II restriction enzymes (Fig. 2). The results showed that the PCR fragments were successfully cloned in the T vector. Sequencing results showed that the length of the inserted fragment was 723 bp, with ATG being the start codon and TAG being the stop codon, encoding 240 amino acids, which had 100% homology to the published nucleotide (access number: AE009419.1).

Amplification, cloning and sequencing of P_{SAG12}: A 1500-bp specific product was amplified using primers designed from the 5′ sequence of the *SAG12* published in the GenBank using the Arabidopsis genomic DNA as the template (Fig. 3), which was consistent with the expected results. After recovery and purification, the fragment was inserted into the pMD18-T vector to obtain the recombinant plasmid pMD18-T and digested with *Bam*H-I and *Nco*-I before sequencing (Fig. 4). DNA sequence analysis showed that the fragment length was 1522 bp and had a homology of 99% with the upstream nucleotide

sequence of the *SAG12* gene (access number: ATU37336) published by the GenBank.

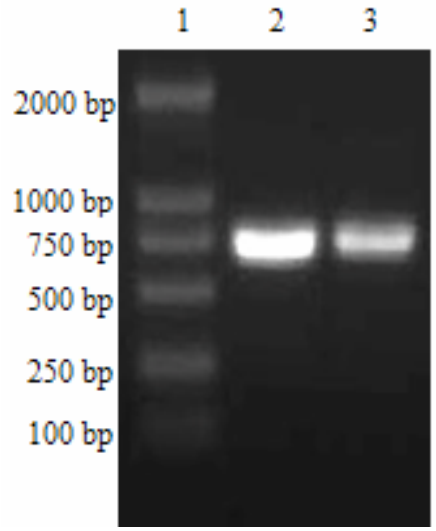

Fig. 1. PCR amplification of ipt gene. 1, DL2000 Marker; 2, 3. The PCR products of ipt gene.

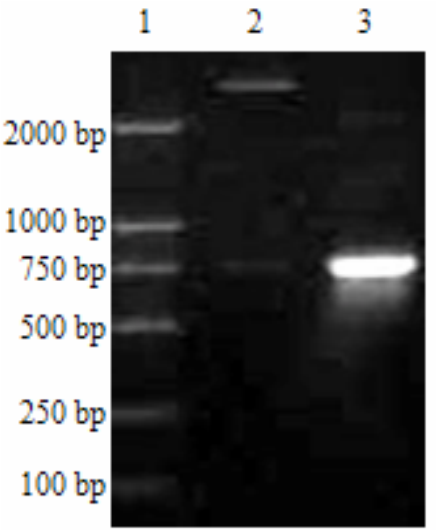

Fig. 2. Identification of recombined pMD18-ipt plasmid digested with *Nco*-I and *Bst*E-II and PCR amplification 1. DL2000 Marker; 2. pMD18-ipt digested by *Nco*-I and *Bst*E-II; 3. PCR amplification using pMD18–ipt vector as a template.

Construction of an intermediate vector pCAMBIA1301-ipt: Recombinant pMD18-ipt and pCAMBIA1301 were doubly digested with *Nco*-I and *Bst*E-II to obtain the ipt and beta-glucuronidase fragments. The ipt gene was inserted into pCAMBIA1301, forming an intermediate vector pCAMBIA1301-ipt. Using the primers P1 and P2, pCAMBIA1301-ipt plasmid fragments with a product size of about 723 bp were obtained, consistent with the previous experiment. A fragment of around 723 bp was released from the plasmid vector after digestion with the restriction enzymes *Nco*-I and *Bst*E-II (Fig. 5), indicating that the ipt gene was successfully inserted into the pCAMBIA1301-ipt.

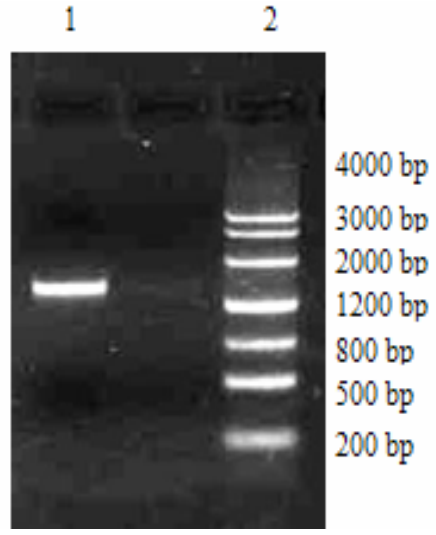

Fig. 3. PCR amplification of P_{SAG12}. 1. The PCR products of P_{SAG12}; 2. DL4000 Marker.

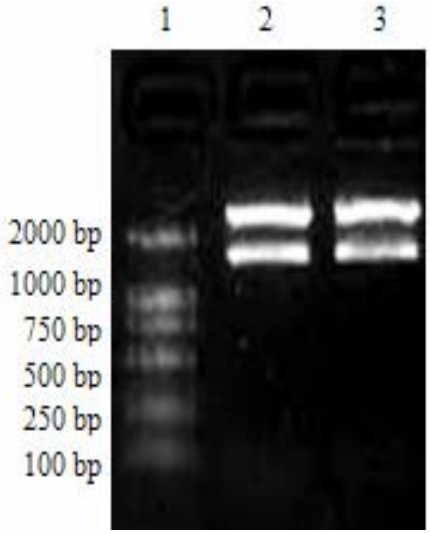

Fig. 4. Identification of recombined pMD18-ipt plasmid digested with *Bam*H-I and *Nco*-I. 1. DL2000 Marker; 2,3. pMD18-SAG12 digested by *Bam*H-I and *Nco*-I.

Construction of plant expression vector pCAMBIA1301-SAG12-ipt: The promoters of SAG12 and the c***auliflower mosaic virus*** 35S genes were isolated by digesting pMD18-SAG12 vector and the intermediate vector pCAMBIA1301-ipt with *Bam*H-I and *Nco*-I. The SAG12 promoter was ligated to the pCAMBIA1301-ipt, yielding the SAG12 promoter–driven plant-expression vector pCAMBIA1301-SAG12-ipt. Using the primers P3 and P4, a fragment of about 1500 bp was amplified from pCAMBIA1301-SAG12-ipt, which was consistent with the previous experiment. After digestion of the plasmid vector by *Bam*H-I and *Nco*-I, fragments with lengths of about 1500 bp were also obtained (Fig. 6). The results from the plasmid experiments, PCR and restriction

enzyme digestion are consistent with the theoretical calculation and show that the expression vectors were successfully constructed. The construction of the plant-expression vector is shown in Fig. 7.

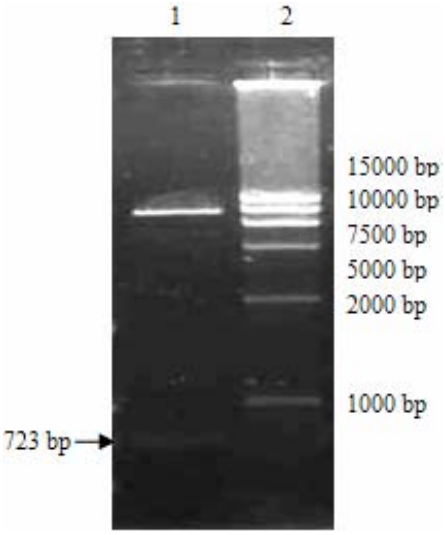

Fig. 5. Identification of recombined pCAMBIA1301-ipt plasmid digested with *Nco* I and *Bst*E-II. 1. pCAMBIA1301-ipt digested by *Nco* I and *Bst*E-II; 2. DL15000 Marker.

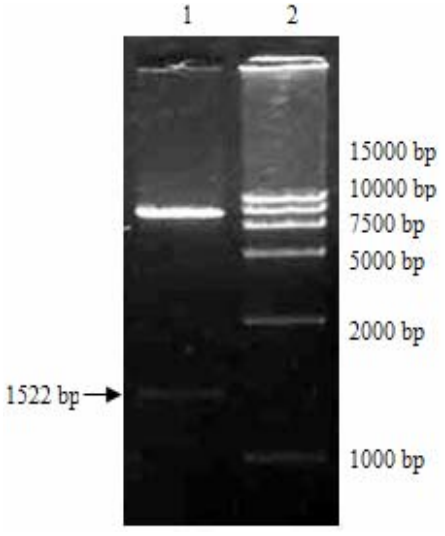

Fig. 6. Identification of recombined pCAMBIA1301- SAG12-ipt plasmid digested with *Bam*H I and *Nco* I. 1. pCAMBIA1301-SAG12-ipt digested by *Bam*H I and *Nco* I; 2. DL15000 Marker.

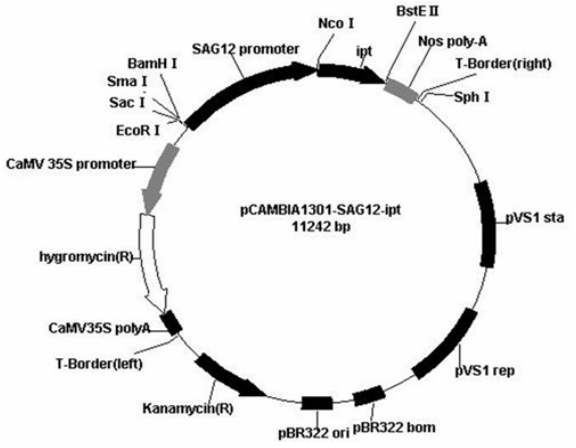

Fig. 7. Construction of binary vector harboring peanut which ipt gene had been fusioned with Arabidopsis SAG12 gene promotor.

***Agrobacterium*-mediated genetic transformation of rice and regeneration of transgenic plants:** Rice calluses were cultivated in differentiation media under light, which developed green points after 1 week (Fig. 8a and 8b). After continuous cultivation for 2 weeks, the green points gradually grew into small buds and further developed into plantlets (Figs. 8c & 8d). A total of 37 transgenic plants were acquired, which were subjected to subsequent detection protocols.

Molecular detection of resistant plants: After the plasmid pCAMBIA1301-SAG12-ipt transformed Japonica Zhonghua 16, the transgenic plants were tested using PCR. The results are shown in Fig. 9, with the expected amplified lengths of 723 bp (ipt gene, lane 4), 1522 bp (SAG12 promoter, lane 7) and 2245 bp (SAG12 promoter plus ipt gene, lane 10); and the positive control plasmids (lanes 3, 6 and 9, respectively), whereas the transgenic plants (negative control) showed no corresponding bands (lanes 2, 5 and 8). Thus, we successfully constructed the plant-expression vectors and used them to transform rice successfully.

Discussion

With the appearance of global warming, premature of food crops became more and more serious (Alejar *et al*., 1995; Hajouj & Gepstein, 1999). Premature was an important factor that lowered crop yields and quality. Therefore, against premature has always been one hotspot of the crop researching. Early leaf senescence was the most important factor which affected crop production in against premature aging study. Major food crops in some areas of China had suffered huge loss caused by early aging (Dong *et al*., 2006). The hot summer drought in three Gorges reservoir area of Chongqing often leads to stigma and seed setting rate activity decreased in the heading and filling stage of rice, and senescence of functional leaves and roots, resulting in high temperature forced-cooked or causing secondary grouting; it also triggers a variety of diseases and insect pests, causing chalky grain rate and chalkiness and decreasing yield and quality. Just in more than 150 million hectares of rice fields in the southeast of Sichuan and Chongqing had almost experienced high forced maturity and leaf senescence every year, especially deteriorated quality of hybrid rice (Shah *et al*., 2011b). In the global context of climate change, natural disasters are more frequent in Chongqing. Although we have promoted breeding of new varieties of anti-drought, its mechanism study has lagged, which was not conducive to the sustainable development of crop production. Therefore, exploration and utilization of the leaf senescence–related functional genes joint with their physiological characteristics and molecular mechanisms of systematic research would provide valuable information for the development of genetic breeding and crop cultivation measures and promote the sustainable development of food crop production , which is of great significance (Ma & Liu, 2009).

Fig. 8. *Agrobacterium*-mediated genetic transformation of rice and regeneration of transgenic plants
(a) Callus from mature embryo (front view), (b) Callus from mature embryo (flank view), (c) Differentiation green point from resistant callus, (d) Young seedling from resistant callus

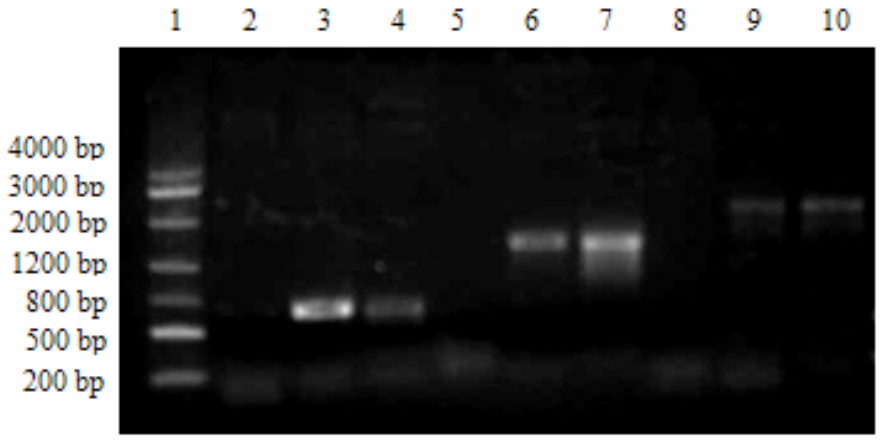

Fig. 9. PCR detection of transgenic rice plants 1. DL, 4000Marker; 2,5,8. Non-transformed plant sample as negative control; 3,6,9. Plasmid DNA as positive control; 4,7,10. Transgenic plant.

Until now, people have done a lot of research about ipt gene transformation (Chan *et al*., 2009; Daskalova *et al*., 2007; Lopez-Noguera *et al*., 2009; Swartzberg *et al*., 2008). The ipt gene in the transformed plant usually causes excessive synthesis of CTK, leading to the abnormal development of plants, including features such as dwarfing of the plants, disappearance of apical dominance, proliferation of buds, incomplete development of roots, dark green colour of the leaves, and so on. The gene-expression specificity was decided by changes in the internal and external environments by the spatial and temporal cell specificity and the biological development process of the individual. The various factors involved in regulating gene expression (especially

at the transcription level) worked through the activity of the promoter in the final transcript (Hu *et al*., 2005; Luo *et al*., 2005). Thus, transformation using the ipt gene and selection of the appropriate promoter in the transgenic plants are both essential to ensure the normal form and function. The promoter must comply with the following requirements: (1) expression in the leaves; (2) expression during leaf senescence; (3) avoiding the over-expression of the ipt gene (Ballester *et al*., 2007; Calderini *et al*., 2007). The SAG12 promoter may thus be an ideal promoter. Zhonghua 16 is one of the rice varieties with a very broad range of favourable characteristics, such as the large amount of growth, well-developed root system, stout stem, glassy rice seeds, and high protein content. Resistance to salinity, drought, lodgings, and insects; and its large-sized pollen, high yield of seeds, and heterosis, are other salient features. Therefore, it can be used to restore the lines of hybrid rice sexes (Serdyuk *et al*., 2007). If there is a delay in the late growth stages of leaf senescence and an extension of photosynthesis time, Zhonghua 16 is undoubtedly capable of accumulating more photosynthetic products.

Conclusions

In this study, we had constructed the plant-expression vector pCAMBIA1301-SAG12-ipt that successfully integrated into the transformed plants, which would provide valuable information for the development of genetic breeding and crop cultivation measures and promote the sustainable development of food crop production.

Acknowledgements

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