Development of an inducible male-sterility system in rice through pollen-specific expression of L-ornithinase (argE) gene of E. coli

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ABSTRACT

In the present investigation, an inducible male-sterility system has been developed in the rice. In order to introduce inducible male-sterility, the coding region of L-ornithinase (argE) gene of E. coli was fused to the Oryza sativa indica pollen allergen (OSIPA) promoter sequence which is known to function specifically in the pollen grains. Transgenic plants were obtained with argE gene and the transgenic status of plants was confirmed by PCR and Southern blot analyses. RT-PCR analysis confirmed the tissue-specific expression of argE in the anthers of transgenic rice plants. Transgenic rice plants expressing argE, after application of N-acetyl-phosphinothricin (N-ac-PPT), became completely male-sterile owing to the pollen-specific expression of argE. However, argE-transgenic plants were found to be self fertile when N-ac-PPT was not applied. Normal fertile seeds were obtained from the cross pollination between male-sterile argE transgensics and untransformed control plants, indicating that the female fertility is not affected by the N-ac-PPT treatment. These results clearly suggest that the expression of argE gene affects only the male gametophyte but not the gynoecium development. Induction of complete male-sterility in the rice is a first of its kind, and moreover this male-sterility system does not require the deployment of any specific restorer line.

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1. Introduction

Exploitation of hybrid vigour in crop plants, which plays an important role in the production of hybrid varieties, is often hampered by the occurrence of self-fertility in the parental lines. In rice, due to its unique floral morphology, controlled cross pollination is difficult to be achieved. Hence, development of cost effective methods to produce hybrid seeds on large scale crossing selected parental lines would greatly contribute to commercial viability of the hybrid varieties. As such, manipulation of male sterility/fertility system is a critical process for hybrid rice breeding to exploit the hybrid vigour. Hybrid rice verities show 15–30% yield increase over the parental lines when grown under similar conditions [1], and are known to respond well under adverse environmental conditions [2]. A number of approaches, viz., emasculation, chemical-induced male-sterility, cytoplasmic and nuclear male-sterility as well as transgenic approaches for pollen abortion, have been attempted to limit self-fertilization in the parents for the development of an effective hybrid seed production system. In plants, the formation of male reproductive system involves several major developmental stages, viz., specification of stamen primordia, production of sporogenous cells, development of tapetum and microspore mother cells, meiosis, formation of free microspores, early and delayed degeneration of tapetum and release of pollen grains [3,4]. Arrest at any of these stages can result in male-sterility involving the loss of ability to produce or release functional pollen grains.

Thus far, in rice, two distinct systems, viz., cytoplasmic male-sterility (CMS) and environment-sensitive genetic male-sterility (EGMS), have been utilized in hybrid seed production [5–7]. Usually, the large scale hybrid seed production in rice is accomplished by employing the three-line system containing cytoplasmic male sterile line (A line), maintainer line (B line) and restorer line (R line). In EGMS, pollen-fertility can be regulated by either day length (photoperiod-sensitive genetic male-sterility; PGMS), or temperature (thermosensitive genetic male-sterility; TGMS) or both (photothermal-sensitive genetic male-sterility; PTGMS) [6]. Various uncontrollable factors, such as change in temperature, duration of day length and duration of the season are known to play...
a major role in the commercial production of high quality hybrid seed.

Transgenic male sterility system serves as an alternative approach for the production of hybrid varieties in crop plants. Through this approach male-sterile plants were developed by ablating floral tissues/ organs using the toxic genes under the control of anther/pollen specific promoters. In the present study, we have employed OSIPA promoter to drive the argE gene for the development of male-sterile transgenic rice plants. The OSIPA promoter contained various cis-regulatory elements, such as AGAAA, GTGA, TGTGA, TGTGTC, TTTC, ACCT and AAAG, involved in the pollen-specific expression of the promoter [8,9]. Functional analysis of OSIPA promoter in the heterologous Arabidopsis and tobacco as well as in rice confirmed its activity at different stages of anther development [8,9]. Mariani et al. [10] demonstrated the development of male sterility in tobacco and brassica by expressing Barnase of Bacillus amyloliquefaciens with the help of tobacco tapetum promoter TA29. Later, various male sterile plants were developed using different tapetum or anther-specific promoters fused to selected candidate genes [11–18]. Furthermore, RNA mediated silencing of genes and antisense technologies were successfully employed for production of male sterile plants [19–23]. Transgenic methods were also developed for the production of conditional male sterile plants in certain crops [22,24]. The advantage of having conditionally sterile parental lines is that it allows them to be maintained as homozygous lines for the sterility trait.

The argE gene of Escherichia coli encodes N-acetylornithinase (NAO; EC 3.5.1.16), which removes the acetyl group from N2-acetylornithine and produces acetylornithine [25]. The enzyme was able to deacetylate N-acetyl-L-phosphinothrin (N-ac-PPT), a nontoxic compound, to produce phosphinothrin (PPT) a toxic compound which kills the cells. The argE gene was successfully expressed in the tapetum tissue of transgenic tobacco plants to develop a system of chemically induced male sterility upon treatment with N-ac-PPT [24]. Chen et al. [26] introduced the argE gene into perennial ryegrass and selectively eliminated the transgenic plants by the application of N-ac-PPT. Transgenic tobacco plants expressing D-amino acid oxidase (DAAO) under control of the TAPI1 tapetum-specific promoter were found male-sterile upon application of the herbicide D-glufosinate [27].

In this investigation, we successfully engineered an inducible male-sterility system in rice by making pollen nonviable without affecting the gynoecium in any way. Transgenic rice plants, expressing argE gene under the control of Oryza sativa indica pollen allergen promoter (OSIPA), showed complete male-sterility after treatment with the N-ac-PPT.

2. Materials and methods

2.1. Isolation of argE gene and construction of plant expression cassette

The coding region of argE gene from E. coli (TOP10 strain) genomic DNA was amplified by PCR using gene-specific primers with BamH I restriction site in both forward 5′-GGATCCATGAAACCATATGCGCA-3′ and reverse 5′-GGATCCTTATGCGCAAAATGTTG-3′ primers. The amplified PCR product was cloned at Smal site of pBSK (+) plasmid and was confirmed by restriction analysis. Further, the recombinant clone was subjected to DNA sequencing. The coding region of argE was subcloned downstream to T7 promoter of pET-21a (+) bacterial expression vector. The pET-21a (+) argE plasmid was mobilized into E. coli BL21DE3 cells and the cells were induced with 0.5 M IPTG to check the expression of argE gene [28]. Later, argE gene was excised from pET21a plasmid and cloned downstream to OSIPA promoter at BamH I site in pCAMBIA1300 vector having hyg gene as a selectable marker. Later, pCAMBIA1300-CaMV35S-hyg-OSIPA-argE vector was mobilized into Agrobacterium (EHA105) by tri-parental mating using helper plasmid pRK2013. Plasmid DNA was isolated from EHA105 cells and digested with Hind III enzyme to confirm the presence of binary vector carrying the gene of interest.

2.2. Agrobacterium-mediated genetic transformation and development of transgenic plants

Seeds of rice cultivar BPT 5204 obtained from the Indian Institute of Rice Research (IIRR), Hyderabad, were used for rice transformation. Agrobacterium-mediated genetic transformation experiments were performed using EHA105 harbouring pCAMBIA1300-CaMV35S-hyg-OSIPA-argE vector according to Ramesh et al. [29]. For the selection of argE putative transformants, the co-cultivated calli were subjected to two rounds of selection on medium containing hygromycin (70 mg/l) for 2 weeks each. Later, the actively proliferating calli were selected and transferred to proliferation medium and then onto regeneration medium as reported earlier [30].

2.3. Molecular analysis of transgenic plants

Genomic DNA was isolated from the argE putative transformants and untransformed control (UC) plants using the method of McCouch et al. [31]. Dual PCR analysis was performed to confirm the putative transformants using four different primers: 5′-ATACCGGATA CGGTGCGATTGTTT-3′ (argE forward) and 5′-TTGGCGGCTATTTTGGTTT-3′ (nos reverse); 5′-ATTTGCGCTCCAAATGTC-3′ (hyg forward) and 5′-GGCTCAACACATGACGAAAC-3′ (polyA reverse). DNA isolated from the UC was used as negative control and intermediate vector was used as positive control. For Southern blot analysis, 15 μg of genomic DNA from argE transformants were digested with EcoR I enzyme, electrophoresed on a 0.8% agarose gel and subsequently transferred to an N+ Nylon membrane (Amersham Biosciences) and fixed by exposing to UV (1200 μJ for 60 s) in an UV cross-linker [28]. The blot was probed with α-32P dCTP employing ready to go random primer DNA labelling kit (Amersham Biosciences) and argE coding sequence was used as a probe.

2.4. RT-PCR analysis

Total RNA was isolated from roots, leaves and anthers of transgenic and UC plants using TRIZOL method (Invitrogen). RT-PCR analysis was carried out employing argE gene-specific 5′-ATACCGGATA CGGTGCGATTGTTT-3′ (argE forward) and 5′-GATTCCGTGGCGCATTC ATAGC-3′ (argE reverse) primers, and the reaction was set to 50 μl containing Tris-HCl (10 mM), KCl (50 mM), MgCl2 (1.5 mM), dNTPs (200 μM each), Taq DNA Polymerase (1.5 units), MMLV reverse transcriptase (2 units), total RNA (~1.0 μg), forward primer (10 PM), and reverse primer (10 PM). The reaction mixture was incubated at 50°C for 30 min and PCR was carried out as described. Amplified PCR products were analyzed by the gel electrophoresis on 1.0% agarose gel.

2.5. Identification of homozygous argE rice lines

To obtain homozygous plants, seeds harvested from argE transgenic plants (T0) were germinated on MS basal medium supplemented with 70 μg/ml hygromycin. After one week of germination, the resistant seedlings (T1) were transferred to pots and allowed to grow to maturity. The harvested seeds of argE transfor-
mants (T₂) were germinated on MS basal medium supplemented with 70 mg/l hygromycin to identify the homozygous lines.

2.6. Chemical synthesis of N-ac-PPT

N-ac-PPT was synthesized according to Chen et al. [26]. Acetic anhydride (21 mM) was added to a solution of PPT (2.1 mM) in dioxane (5.0 ml) and water (5.0 ml), and the resulting mixture was stirred at room temperature overnight. After removal of the solvent, the residue was dried in vacuum to determine the product yield. The purity of the product was tested by Mass spectral analysis.

2.7. Analysis of argE transgenic plants

In order to confirm the effect of argE protein, different parts of transgenic and UC plants, viz., leaves, roots, anther/pollen and gynoecium were tested for their sensitivity to N-ac-PPT. Both transgenic and UC plants were treated with N-ac-PPT adopting two methods namely spraying and irrigation with water. In one method, plants of about 100–110 days-old (early panicle development stage) were sprayed with two concentrations of N-ac-PPT (0.05 mg/ml and 0.075 mg/ml) once/twice (with a gap of 3 days) and allowed to grow to maturity. An amount of 25 ml of N-ac-PPT solution was sprayed on each plant. In another method, plants of 80–90 day old (before panicle primordium initiation) were treated with the three concentrations of N-ac-PPT (0.05, 0.1 and 0.2 mg/ml) once/twice (with a gap of 10 days) and allowed to grow to maturity. Through irrigation method, 50 ml of N-ac-PPT solution was supplied for each plant.

2.8. Pollen viability analysis

Pollen grains of different argE transgenics and UC plants were tested with Alexander staining [32] to distinguish fertile and sterile pollen. Pollen germination assay was carried out using the protocol described by Han et al. [33]. Pollen grains of transgenics and UC plants were placed on a petri plate containing the pollen germination medium, supplemented with 1 mM CaCl₂, 1 mM KCl, 0.8 mM MgSO₄, 1.6 mM H₂BO₃, 30 μM CaSO₄, 0.3% 2-N- Morpholino ethanesulfonic acid, 10% sucrose and 12.5% polyethylene glycol, and incubated at the room temperature for 2 h. Stained and germinated pollen grains were observed under OLYMPUS BX41 microscope.

2.9. Fertility analysis of female reproductive system in argE transgenics

Fertility of the argE transgenic plants after treating with N-ac-PPT was determined by allowing the pollination from the UC plants. Manual pollination was carried out by placing the argE transgenic male-sterile plants obtained after N-ac-PPT treatment and UC plants side by side in the glass house. The pollen of UC plants was dusted by tapping the panicles onto the top of argE transgenic plant’s panicles repeatedly for three consecutive days (once per day) during the morning hours after extrusion of stigma from the spikelets.

3. Results

3.1. Isolation of argE gene and construction of plant expression cassette

The nucleotide sequence analysis of the PCR product was found to be identical to that of argE gene (GenBank Acc. Nos. X55417.1 GI: 40956). The gene contained 1152 bp coding sequence and encodes a polypeptide of 384 amino acids. Expression analysis revealed the presence of ~42 kDa polypeptide in induced E. coli cells corresponding to the argE product (Supplementary Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2016.12.001). The restriction analysis of plasmid construct pCAMBIA-CaMV35S-hyg-OSIPA-argE revealed a 2.13 kb size fragment corresponding to the size of OSIPA promoter and argE gene (Supplementary Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2016.12.001). The OSIPA promoter expressed exclusively in the developing anthers, and its maximum expression started from stage 8 onwards and continued upto anthesis. However, it showed no expression in the lemma and palea as evidenced by the histochemical GUS analysis (Supplementary Fig. S2 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2016.12.001).

Fig. 1. (A) Restriction map of T-DNA region of the pCAMBIA-CaMV35S-hyg-OSIPA-argE. (B) Southern blot analysis of argE transgenic plants. Genomic DNA was digested with EcoRI enzyme and probed with argE gene coding sequence. P: represents positive control, UC: represents untransformed control, 1–6 represent six different argE transgenic lines. (C) RT-PCR analysis of argE transgenics. RNA was isolated from the stage 12 of the anther development (late pollen stage). M: 1 kb DNA marker, P: Positive control, Lanes 1 & 2: RNA from roots and leaves of argE 9–6, Lanes 3 & 4: RNA from anthers of argE 9–6 & argE 25–3 transgenic lines.
3.2. Development of transgenics and their confirmation by molecular analysis

A total of 24 putative transformants were obtained from 3249 infected calli employing pCAMBIA-CaMV35S-hyg-OSIPA-argE construct (Fig. 1A). Dual PCR analysis of transformants, using argE forward – nos reverse and hyg forward – pO0K reverse, revealed the amplification of two bands of 1.1 kb and 527 bp corresponding to argE and hyg genes, respectively, while UC failed to show any amplification (Supplementary Fig. S3 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2016.12.001). When the genomic DNA of argE transgenic lines was digested with EcoR I and probed with argE gene coding sequence, it showed a hybridizable signal at >4.0 kb region, whereas UC showed no such hybridization (Fig. 1B).

3.3. RT-PCR analysis of argE transformants

RT-PCR analysis was carried out to analyze the tissue-specific expression of argE gene driven by the OSIPA promoter. The results revealed the presence of argE gene transcript (~700 bp) exclusively in the anthers of transgenic plants (Fig. 1C).

3.4. Identification of homozygous argE rice lines

A 3:1 phenotypic ratio of resistant and susceptible seedlings was observed when T1 seeds were germinated on MS medium supplemented with hygromycin (70 mg/l). All the resistant seedlings were grown to maturity and T2 seeds were collected. Further germination of T2 seeds on MS basal medium supplemented with hygromycin (70 mg/l) showed 100% germination in some of the progenies. These lines were identified as homozygous and used for subsequent analysis (Supplementary Fig. S4 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2016.12.001).

3.5. Evaluation N-ac-PPT effect on argE transformants

To confirm the effect of N-acetyl-l-ornithinase, different argE transformants of rice were tested for their sensitivity to N-ac-PPT. Alexander staining was performed before and after N-ac-PPT treatment for both argE transgenics and UC plants to analyze the percentage of fertile and sterile pollen grains. Around 5000 pollen grains from each line were stained and observed under the microscope. Untreated pollen of UC and argE transformants showed magenta-red colour which indicates the fertile nature of pollen (Fig. 2). In contrast, pollen from transgenic lines exhibited 100% blue-green coloured pollen after N-ac-PPT treatment, a characteristic feature of sterile pollen (Fig. 2). Pollen of UC treated with N-ac-PPT showed magenta-red colour (Fig. 2). On the other hand, gynoecium of all argE transformants after N-ac-PPT treatment stained magenta-red colour similar to UC plants.

Pollen germination assay was carried out before and after N-ac-PPT treatment for both argE transgenics and UC plants to test the germination ability of pollen grains. More than 1000 pollen grains from each transgenic male sterile line and UC pollen grains were employed for germination analysis. Untreated pollen of UC and argE transformants showed 100% pollen germination (Fig. 3). In contrast, pollen from transgenic lines failed to show germination after N-ac-PPT treatment, while the pollen grains of UC plants treated with N-ac-PPT showed 100% germination similar to that of untreated pollen grains (Fig. 3).

with irradiation, and K & L: Gynoecium of UC and argE transformants treated with N-ac-PPT (0.2 mg/ml) with irrigation.
Homoygous argE transgenic plants sprayed with N-ac-PPT (0.05 mg/ml and 0.075 mg/ml) showed differential pollen sterility. Panicles of transgenic plants sprayed with 0.05 mg/ml of N-ac-PPT either once/twice exhibited about 20–30% sterile pollen grains. Whereas panicles of argE transgenants sprayed with 0.075 mg/ml N-ac-PPT twice with a gap of three days showed 100% sterile pollen grains in argE 3-2, argE 9-6 and argE 25-3 lines (Table 1). The remaining 4 lines viz., argE 4-6, argE 14-3, argE 21-6 and argE 24-2 disclosed incomplete sterility (62.2% to 91.4%). However, the UC plants sprayed with the N-ac-PPT showed complete fertile pollen grains (Fig. 2, Table 1).

In the second method (irrigation), all the 7 lines, viz., argE 3-2, argE 4-6, argE 9-6, argE 14-3, argE 21-6, argE 24-2 and argE 25-3 that were treated once/twice with 0.05 mg/ml and 0.1 mg/ml of N-ac-PPT showed majority of fertile pollen with a low frequency (5% to 15%) of sterile pollen. Whereas, transgenic lines of argE 3-2, argE 9-6 and argE 25-3 treated twice with 0.2 mg/ml N-ac-PPT showed complete (100%) sterile pollen while the remaining four lines, viz., argE 4-6, argE 14-3, argE 21-6 and argE 24-2 displayed about 62% to 92% sterile pollen (Table 2). All the UC plants treated with different concentrations of N-ac-PPT exhibited normal fertile pollen grains. Both the methods gave identical results and proved to be effective in inducing male sterility in rice. In argE transgenic plants, after treatment with N-ac-PPT, visible morphological changes like small, shrunken, brown coloured anthers were observed. In contrast, other parts of plant, viz., leaves, roots, lemma, palea and gynoecium of argE transformants were normal and similar to that of UC plants (Figs. 2, 4 and Supplementary Fig. S5 in the online version at DOI: http://dx.doi.org/10.1016/j.plantsci.2016.12.001).

3.6. Grain filling ability of argE transgenants after N-ac-PPT treatment

To observe the grain filling ability of UC and argE transgenants, they were treated with N-ac-PPT and allowed to grow to maturity. UC panicle treated with N-ac-PPT showed normal grain filling but argE transformants failed to show any grain filling (Fig. 5). No differences were observed between N-ac-PPT treated and untreated control plants in their grain filling ability.

3.7. Fertility analysis of female reproductive system in argE transgenics

The argE transgenic lines which showed complete male sterility, after N-ac-PPT treatment, were allowed to pollinate with the UC pollen to test the female fertility of these plants. The results revealed that the complete male sterile lines of argE developed fertile seeds after pollination from the UC plants (Fig. 6).

4. Discussion

Male sterility facilitates the control of self-fertility in crop plants and thus helps in the production of hybrid seeds. Adoption of new strategies for the development of male-sterile plants is essential for...
Fig. 4. Effect of N-acetyl-PPT on male fertility of argE transgenic rice plants. (A) Plants treated with N-acetyl-PPT by spraying topically (0.075 mg/ml). (B) Plants treated with irrigation (0.2 mg/ml). UC: Untransformed control showing panicles with normal anthers, 9-6 & 25-3: Two argE transformants showing panicles with sterile anthers.

Fig. 5. Seed setting ability of argE transgenic rice plants after treating with N-acetyl-PPT. (A) UC plants and argE transgenic plants were treated with N-acetyl-PPT by spraying (0.075 mg/ml) and (B) with irrigation (0.2 mg/ml). Later, they were allowed to self pollinate. UC: Panicle of untransformed control showing normal seed setting. 9-6 & 25-3: Panicles of two argE transformants showing the failure of seed setting.
Table 2
Effect of N-ac-PPT on pollen fertility of argE rice transformants after treatment with irrigation.

<table>
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<tr>
<th>Transgenic line</th>
<th>No. of pollen grains stained</th>
<th>No. of pollen grains sterile</th>
<th>No. of pollen grains fertile</th>
<th>Pollen grains sterile (%)</th>
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<td>argE-3-2</td>
<td>5000</td>
<td>5000</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>argE-4-6</td>
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<td>3740</td>
<td>1260</td>
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<tr>
<td>argE-5-6</td>
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<td>5000</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
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<td>5000</td>
<td>3100</td>
<td>1900</td>
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</tr>
<tr>
<td>UC</td>
<td>5000</td>
<td>0</td>
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</table>

Fig. 6. Seed setting ability of argE male-sterile rice lines after pollination with UC plants. The argE transgenic plants after treating with N-ac-PPT were allowed to pollinate from the UC plants. (A) UC plants and male-sterile argE lines obtained by spraying N-ac-PPT topically and (B) by irrigation. UC: Untransformed control, 9-6 & 25-3: Two argE transgenic plants.

the production of hybrid varieties in major crop plants. Transgenic male-sterility system has been successfully developed in different plant species through genetic engineering approaches utilizing various candidate genes [34]. In this report, we present an efficient method to produce completely male-sterile transgenic rice lines employing argE gene fused to a pollen specific promoter of OSIPA. Thus far, various cytotoxic genes have been employed for the production of male-sterile transgenic plants in rice crop. However, there is no report regarding the exploitation of argE gene for the development of an inducible male-sterility system in rice.

The enzyme N-acetyl-ornithine deacetylase, which plays an important role in the arginine biosynthetic pathway, also deacetylate N-acetyl-PPT and produce PPT, owing to its broad range of substrate specificity. Accordingly, the argE gene expressing anthers can convert N-acetyl-PPT to PPT. The resultant PPT causes damage to the developing anthers due to its toxic nature and results in conditional male sterility. The N-ac-PPT proved to be a suitable inducer substance and when applied on the leaves it is distributed to different parts of the plant and is accumulated mainly in the upper parts of the plant [35]. The N-ac-PPT is non-phytotoxic and remains unchanged for more than fourteen weeks in the plant [36]. The constitutive expression of the argE gene did not lead to changes in the growth, regeneration and reproductive capacity of the plants. However, in the presence of its substrate N-ac-PPT, a massive destruction of the cells expressing the transgene could be detected. As such, the argE gene is more suitable to induce specific cell death in plants which are healthy in the absence of the inducer [24].

In our earlier studies, we have demonstrated that the OSIPA promoter could express exclusively in the developing anthers of Arabidopsis, tobacco and rice, and not in any other part of the plant [8,9]. Based on these results, we have employed OSIPA promoter to drive the expression of argE gene specifically in the pollen so as to induce male sterility. Results from the Southern blot analysis of transformants clearly demonstrated the unequivocal integration of argE gene predominantly as single copy in the genomes of different transgenic plants. Furthermore, RT-PCR analysis confirmed the tissue specific expression of argE in the anthers of transgenic plants.

The evaluation of N-ac-PPT activity on homozygous argE transgenic plants was conducted in two ways either by topical application (spraying) or by treating plants along with water (irrigation). The ideal time period for direct spraying is during the emergence of panicles, i.e., 100–110 days, and for treating with water is 80–90 days, before the emergence of panicles. The topical application of 0.075 mg/ml N-ac-PPT is adequate to induce...
male-stereotype in the transgenic lines, while 0.2 mg/ml was required to cause complete male-stereotype in the transgenic plants given with water. The concentration of N-ac-PPT used in the direct spraying experiments was 40- and 67-fold lesser than that used in rye grass [26] and tobacco [24], respectively. Both the methods exhibited identical results without any variation owing to the efficiency of argE in inducing male-stereotype in rice. Visible morphological changes like small, shrunk, brown coloured anthers were observed only in transgenics that were treated; whereas, untreated transgenics were similar to that of control plants indicating that the argE inducible system does not cause any change in the plant.

The flowers of transgenics were found normal with fertile pollen grains before N-ac-PPT treatment. However, after its treatment the transgenic plants failed to self-fertilize and develop seeds. The absence of seed setting in the panicles of argE transgenic plants, after N-ac-PPT treatment, amply indicates the male-stereile nature of the plants. To demonstrate the female fertility of N-ac-PPT treated transgenic plants, cross pollination was allowed with the untransformed control plants. Normal seeds were obtained from the above crosses similar to that of control plants demonstrating that the female fertility is not affected by the N-ac-PPT treatment. These results clearly indicate that the expression of argE gene affects only the male gametophyte but not the gynoecium development in the transgenic plants treated with N-ac-PPT. Although the successful development of male sterile-lines was achieved by different methods, viz., expressing cytotoxic genes fused with anther-specific promoters [37–40], mitochondria-targeting [41], RNAi [19] and manipulation of metabolite levels [42], the use of a specific restorer line is essential for restoration of fertility.

In rice, induction of complete male-stereotype by expressing argE gene under the control of pollen-specific promoter OSIPA has been accomplished for the first time. The present male sterility system developed in the rice has unique advantages, such as the choice of induction, specificity in application, low amount of N-ac-PPT substrate, pollen-specific expression of transgene, normal female fertility, normal seed setting after cross pollination and nonuse of specific restorer line compared to earlier male sterility systems.

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