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An efficient method for organic acetylation and use of DL-phosphinothricin as a negative selection agent in *argE* transgenic rice

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ABSTRACT

We present an efficient method for the production of *N*-acetyl-L-phosphinothricin (N-AcPt) from commercial DL-phosphinothricin (DL-PPT) by organic acetylation for use as a negative selection agent (NSA) that induces cell death in *argE* transgenic rice. DL-PPT was efficiently converted into N-AcPt with tetrahydrofuran (THF) and acetic anhydride (Ac_2O). Chemical changes were confirmed using NMR and ATR-FTIR analyses. DL-PPT was toxic but N-AcPt did not show cytotoxic effects on leaf discs or seed germination of wild-type rice. Conversely, in *argE–hpt* transgenic rice, non-toxic N-AcPt showed the negative selection (NS) effect by inducing cell destruction in leaf discs and restricting seed germination. For inducing NS, $\geqslant 0.1$ mg ml^{-1} and $\geqslant 0.5$ mg ml^{-1} of N-AcPt were effective in leaf and seed assays, respectively. Further, the NS effect occurred faster in the leaf assay compared with the seed germination assay, again indicating the leaf assay was a more sensitive indicator of N-AcPt as an NSA to *argE* transgenic rice than the seed germination assay. This negative selection approach could be useful for the development of selectable marker free transgenic plants in the economically important monocot species and its commercialization for multiple gene transformation.

1. Introduction

The selection of transgenic cells or tissues is a critical factor for obtaining the desired transgenic plants. Depending on the purpose of the transformation, either positive selection (PS) or negative selection (NS) has been employed and therefore selection agents (SA) and selectable marker (SM) genes are used differently. The growth of transformed cells or tissues is promoted in the presence of SA by the expression of an SM gene in PS, but is inhibited in NS [1,2]. In PS systems, cytotoxic substances, such as antibiotics (e.g., hygromycin), herbicide ingredients (e.g., phosphinothricin: PPT), and the genes conferring resistance to them (e.g., *hpt* [3] and *bar* or *pat*) [4], have been commonly used as SA and positive selectable marker (PSM) genes, respectively. Although the use of PSM genes has had success, efforts have been made to reduce their use since they remain within the transgenic plants, and thus affect the environment [5,6] and human health [1,7]. Also, the use of the same PSM gene would be limited for the addition of further transgenic traits [8].

Conversely, in the NS system, the *Iaah*, *codA*, *argE*, and *P450* genes served as negative selectable marker (NSM) genes to convert non-toxic naphthalene acetamide, 5-fluorocytosine (5-FC), *N*-acetyl-L-phosphinothricin (N-AcPt), and proherbicide R7402 into cytotoxic naphthalene acetic acid [9,10], 5-fluorouracil (5-FU) [11–13], L-phosphinothricin (L-PPT) [14,15], and herbicide R7402 [13,16,17], respectively.

The *argE* gene has been useful as an NSM candidate for inducing the selective destruction of plant tissue. In bacteria, the *argE* gene, the structural gene involved in the biosynthetic pathway of arginine from glutamate, encodes *N*-acetylornithine (NAO) deacetylase (named *argE* deacetylase), which converts NAO into ornithine by deacetylation [18–20]. *ArgE* is also associated with the biosynthesis of cytotoxic L-PPT from the non-toxic precursor N-AcPt, because the gene product has a broad spectrum of substrates [19]. Bacterial *argE* led by a tapetum-specific promoter was first expressed in transgenic tobacco (*Nicotiana tabacum* L.) to induce male sterility [14].

Agrobacterium-mediated co-transformation, as well as a site-specific recombination system, is a promising strategy for developing SMF plants [2,6]; however, laborious PCR analysis is required to select SMF transgenic progenies [1,8]. In contrast, the use of the NSM gene in combination with appropriate substances enables the investigation of SMF transgenic plants without the need for molecular analysis. Park et al. proposed an efficient method by using co-transformation combined with *codA* as an NSM gene to

Abbreviations: PS, positive selection; NS, negative selection; PSA, positive selection agent; NSA, negative selection agent; SM, selectable marker; PSM, positive selectable marker; NSM, negative selectable marker; SMF, selectable marker free; PPT, phosphinothricin; N-AcPt, *N*-acetyl-L-phosphinothricin.

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develop SMF transgenic tobacco [8]. In another study, Lee proved that the utilization of organically acetylated N-AcPt was useful for NS in bacterial *argE* transgenic tobacco [21]. Despite its usefulness as NSM, the *argE* gene combined with N-AcPt has been used to induce male sterility in tobacco.

The objectives of this study were to develop and evaluate the potential of an efficient method for the production of N-AcPt using the organic acetylation of commercial L-PPT to provide a substrate for the *argE* encoded enzyme. The NS system was made practical by applying this product to *argE–hpt* transgenic rice produced by *Agrobacterium*-mediated transformation.

2. Materials and methods

2.1. Production of arge–hpt transgenic rice and molecular analysis

Agrobacterium tumefaciens-mediated transformation using a modification of the method used by Hiei et al. [22] was used to develop *argE–hpt* transgenic rice. Yellow and compact embryogenic calli were induced from mature japonica rice seeds (cv. Ilmibyeo) and transformed using *A. tumefaciens*, strain AGL1 carrying pCAMBIA1300 harbored with *argE* and *hpt* genes, which encode NAO deacetylase and hygromycin phosphotransferase, respectively. Each gene was constructed independently with a CaMV35S promoter in the same T-DNA region as that of the vector. Transgenic plants were selected and regenerated in the presence of 50 μg ml^{-1} hygromycin and T1 seeds were harvested from each plant.

The total genomic DNA was extracted from fresh leaves of T0 rice plants and from hygromycin-selected T1 progenies using the modified CTAB method [23]. *ArgE* and *hpt* transgenes were identified by PCR amplification reaction with the synthetic oligonucleotide primers as follows: *argE* forward (5′-GGGATCCATGAAAAACAA ATTACCGCCATT-3′) and *argE* reverse (5′-GGAGCTCTTAATGCCAGC AAAAATGGTGA-3′), *hpt* forward (5′-ATCCTTCGCAAGACCCTTCCT-3′) and *hpt* reverse (5′-GGTGTCGTCCATCACAGTTTG-3′). A thermo-cycler (PTC-200 DNA Engine, Bio-Rad Co.) was used for PCR amplification under the following conditions: pre-denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, extension at 74 °C for 60 s, and the final extension at 74 °C for 7 min.

Total RNA was extracted from young leaves of T1 plants, selected by hygromycin, using an Easy-Spin™IIp Plant RNA Extraction Kit. The first strand of cDNA from 1 μg of total RNA was synthesized using a Power cDNA Synthesis Kit (iNtRON Biotechnology Inc, Kyunggi-Do, Korea) with the oligo(dT)-primer and reverse transcriptase from an avian myeloblastosis virus (AMV). *OsActin* (Genbank accession no. AB047313) was used as an internal reference gene with the primer as follows: *OsActin* forward (5′-ATGGCTGACGCCGAGGATAT-3′) and *OsActin* reverse (5′-AGG AGCCAAGGCAGTGATCT-3′). RT-PCR amplification was performed to identify the expression of transgenes and the reference gene under the following conditions: pre-denaturation at 94 °C for 5 min, 28 cycles (30 cycles for reference gene) of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, initial extension at 74 °C for 30 s, and the final extension at 74 °C for 7 min. Amplified products in 20 μl were separated on 1.2% (w/v) agarose gel in a 0.5X TBE (Tris–borate–EDTA) electrophoresis buffer along with a 1 kb DNA ladder marker.

2.2. Preparation of N-AcPt from DL-PPT

Commercial DL-phosphinothricin (DL-PPT, glufosinate ammonium) was chemically acetylated to prepare *N*-acetyl-phosphinothricin (N-AcPt) by modifying the methods previously used by Lee [21]. DL-PPT (250 mg, Duchefa Co., Netherlands) was dissolved in 5 ml distilled water and completely reacted, first with 20 ml tetrahydrofuran (THF) and then with 20 ml acetic anhydride (Ac_2O) by stirring in an ice-bath for 8 h. The synthetic product was concentrated by evaporation at 53–55 °C under reduced pressure for 30 min, then at 78–82 °C for 3 h.

2.3. NMR and ATR-FTIR spectroscopy measurements

To identify the chemical changes, N-AcPt was analyzed from ^1H spectra using nucleic magnetic resonance (NMR, Varian Gemini 200 MHz) with deuterium oxide (D_2O) as the solvent. The chemical changes occurring of the functional group of DL-PPT compared to that of N-AcPt were confirmed using attenuated total reflectance Fourier transform infrared (ATR-FTIR, Vertex 70 spectrometer (Bruker Optics Inc, Ettlingen, Germany)) spectroscopy. Data was acquired in reflectance mode over the range of 4000–600 cm^{-1} at 4 cm^{-1} increments and processed using Bruker Optics Opus software.

2.4. Assay for cytotoxic and NS effect of N-AcPt

To identify cytotoxic and NS effects, commercial DL-PPT and its acetylated product, N-AcPt, were applied to seeds and leaf discs of non-transgenic wild type (WT) japonica rice (cv. Ilmibyeo) and *argE–hpt* T1 transgenic plants. Young leaf discs of WT and hygromycin selected T1 plants were placed onto a sheet of sterilized filter paper on a 24-well culture plate and treated with 2 ml of N-AcPt (0.05–2 mg ml^{-1}) and/or DL-PPT (5 μg ml^{-1}) solutions containing 0.1% (v/v) of tween 20. The culture plates were covered with lids, sealed with parafilm to prevent dehydration, and incubated at 25 °C in darkness for 5 d.

In seed assays, surface sterilized rice seeds were planted on a hormone-free semi-solid MS medium supplemented with either various concentrations of N-AcPt (0.05–1 mg ml^{-1}) or DL-PPT (5–15 μg ml^{-1}), respectively. Seeds were cultured on the media at 25 °C and 14 h photoperiod for 12 d after which the germination percentages were calculated. At 12 d, PCR amplifications of the *argE* and *hpt* transgenes were conducted with the live leaf tissue of the germinated plants to confirm the NS effect of N-AcPt on *argE* transgenic plants in response to the concentrations.

3. Results

3.1. Development of arge–hpt transgenic rice and molecular analysis

Since *A. tumefaciens* strain AGL1 carried the vector constructed with both *argE* and *hpt* transgenes under the control of CaMV35S in the same T-DNA region, the transgenic plants that showed hygromycin resistance must have *argE* gene (Fig. 1A).

Transgenes were identified from PCR analysis in 99 out of 102 T0 plants (Fig. 1B-top) and in all hygromycin selected T1 plants (Fig. 1B-bottom). The amplified lengths of *argE* and *hpt* genes were 1,166 and 650 bp, respectively. Further, mRNA expressions of both transgenes were confirmed in 13 hygromycin-selected T1 plants by RT-PCR (Fig. 1C). *OsActin*, the reference gene, was expressed in all T1 and non-transgenic plants; expression of the transgenes, *argE* and *hpt*, was observed only in T1 progenies. Thus, the successful integration, inheritance, and expression of the transgenes were confirmed by molecular analysis, PCR with genomic DNA and RT-PCR with mRNA.

3.2. N-Acetylation of DL-PPT and its NMR and ATR-FTIR analysis

For use as NSA, N-AcPt was prepared using organic acetylation from a commercial DL-PPT. PPT and N-AcPt were confirmed

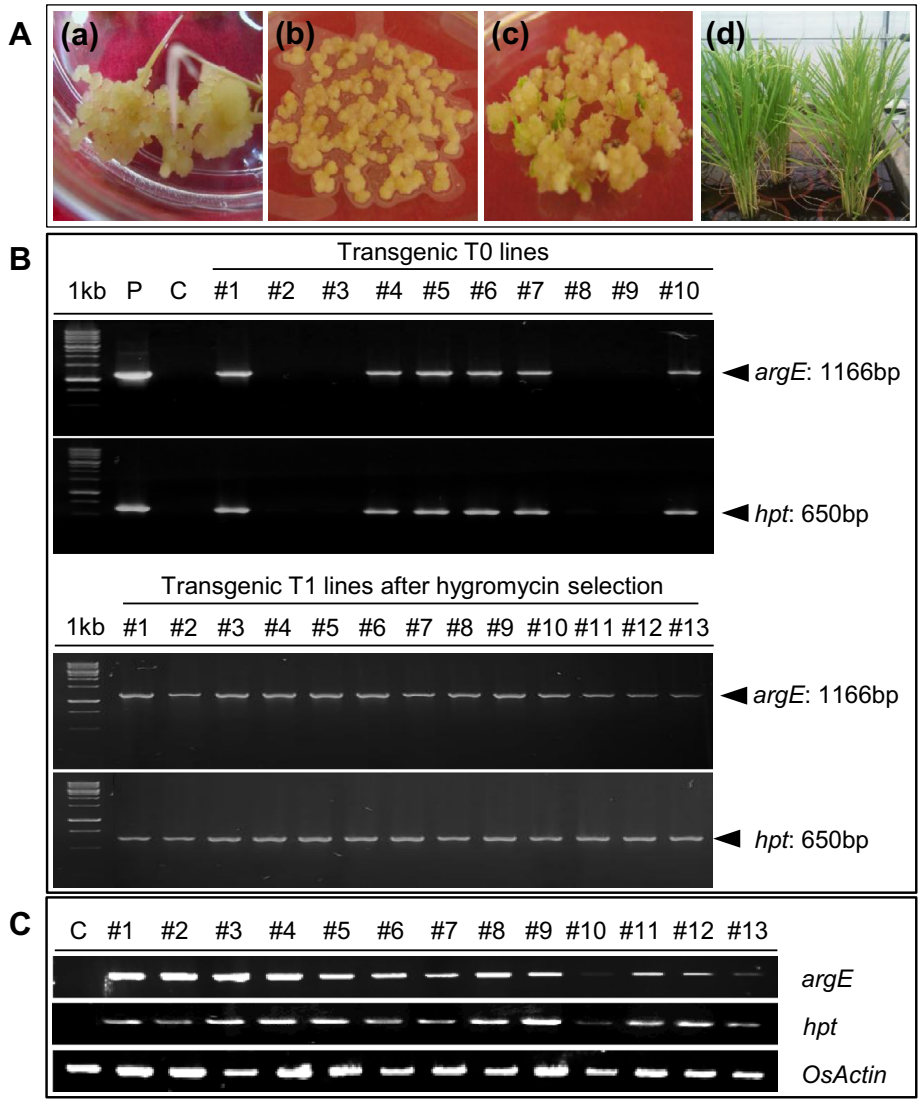

Fig. 1. Production and molecular analysis of *argE–hpt* transgenic rice. (A) *Agrobacterium*-mediated transformation. (a) Callus induction on N6 medium supplemented with 2 μg ml^{-1} of 2,4-D. (b) Co-culture of the calli with *Agrobacterium* AGL1 carrying *argE* and *hpt* gene. (c) Regeneration of hygromycin (50 μg ml^{-1}) selected calli. (d) T0 plants growing in submerged soils in a greenhouse. (B) PCR analysis of *argE–hpt* transgenic T0 (a) and T1 plants (b). The *argE* and *hpt* genes were detected with 1166 and 650 bp in T0 and hygromycin selected T1 plants. P: plasmid positive control, C: Ilmibyeo, WT control. (C) mRNA expression of the transgenes, *argE* and *hpt*; the control gene, *OsActin*, was analyzed by RT-PCR in T1 plants, C: Ilmibyeo, WT control, 1–13: T1 plants.

(Fig. 2A) as metabolic intermediates in the biosynthesis of bacterial BA [30–32]. When the acetylation reaction was complete, the temperature of the mixture decreased.

Based on NMR analyses, the NH_2 of DL-PPT (Fig. 2B, left) was efficiently changed to HNAc ($HNCH_3O$) by adding THF and Ac_2O (Fig. 2B, right). The spectra of DL-PPT and N-AcPt were as follows: DL-PPT; ^{1}H NMR (200 MHz in D_2O) δ ppm 3.70 (t, J = 5.8 Hz, 1H, CH), 1.5–2.1 (m, 4H, PCH_2CH_2), 1.17 (d, J = 13.5 Hz, 3H, PCH_3) and N-AcPt; ^{1}H NMR (200 MHz in D_2O) δ ppm 4.33 (t, J = 5.1 Hz, 1H, CH), 1.7–2.1 (m, 4H, PCH_2CH_2), 1.92 (s, 3H, CH_3CON), 1.39 (d, J = 13.9 Hz, 3H, PCH_3). The CH (#1) peak on the right spectra was shifted further downfield (from 3.7 to 4.4 ppm) due to the increasing deshielding from the two carbonyl groups on the carbon. This proves the conversion of the primary amine to the secondary amine (the acetamide).

The ATR-FTIR spectra of DL-PPT (black) and N-AcPt ([1]red) are shown in Fig. 2C. In the DL-PPT spectra, the primary amine N—H stretch should be observed at the range of 3400–3300 cm^{-1}, with the NH_2 bend at 1633 cm^{-1}. Conversely, while the amide group in N-AcPt should show a C=O stretch at 1640 cm^{-1} and an N—H stretch in the region of 3200–3140 cm^{-1}, the two spectra were not clearly distinguished in these frequencies. However, differences were evident between the C—N stretch of primary amines in the range of 1230–1030 cm^{-1} and of secondary amides with the N—H bend (CHN combination) at 1563 cm^{-1}. In addition, the C—H deformation of the acetyl methyl group at 1380 cm^{-1} supports the result obtained from NMR analysis, suggesting that the NH_2 group of DL-PPT was changed to CH_3CO-N of N-AcPt.

3.3. Assay for cytotoxic effect of N-acetylated PPT on rice

In the seed assay, all non-transgenic seeds germinated on MS medium were supplemented with N-AcPt and grew up to 14 cm at 14 d after treatment (Fig. 3A-a). However, the seeds did not germinate even at the lowest concentration (5 μg ml^{-1}) (Fig. 3A-b).

Non-cytotoxic effects were shown on leaf discs treated with different concentrations of N-AcPt, even at the highest

[1] For interpretation of color in Fig. 2, the reader is referred to the web version of this article.

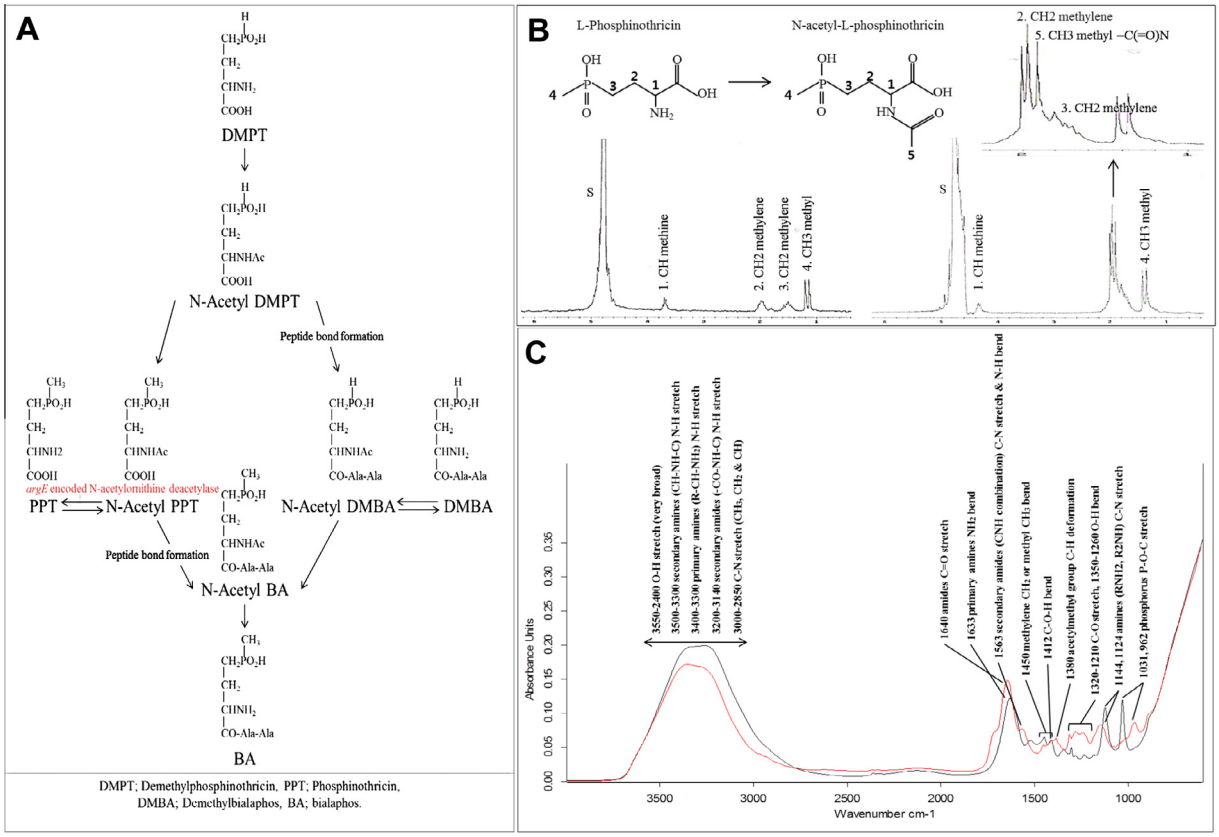

Fig. 2. Production of non-cytotoxic N-AcPt from cytotoxic DL-PPT. (A) Biosynthetic pathway from DMPT to BA. (B) ^{1}H NMR spectra of DL-PPT (left) and *N*-acetylated PPT (right). (C) Spectra of DL-PPT and *N*-acetylated PPT by ATR-FTIR analysis.

concentration of 2 mg ml^{-1} which was 400 times higher than that of DL-PPT (Fig. 3B-a). However, at 5 μg ml^{-1} of DL-PPT, the leaf tissues began to change in color from green [2]to yellow at 4–5 d after treatment, and had senesced at 10 d (Fig. 3B-b). While DL-PPT was cytotoxic, N-AcPt at the concentrations used was non-cytotoxic to leaf discs and seed germination due to the successful *N*-acetylation of DL-PPT.

3.4. Effect of negative selection on argE transgenic plants

The NS effect of N-AcPt was proved with T1 progenies obtained from *argE–hpt* T0 plants (Fig. 4). In the leaf assay, the leaf colors of the hygromycin-selected T1 plants were not significantly changed in the treatment with distilled water (DW) and in combination with 0.1% (v/v) tween 20 for 5–10 d (Fig. 4A). However, cell damage and a marked change in the color of the leaf discs from green[3] to yellow were observed in all concentrations of N-AcPt (0.1–2 mg ml^{-1}) at 4–5 d after treatment. This indicates that the NS effect was successfully expressed on the *argE* transgenic leaves.

Also, in the seed germination assay, the NS effect of N-AcPt was confirmed with T1 progenies (Fig. 4B). From three independent and replicated experiments, all non-transgenic seeds steadily germinated in the presence of N-AcPt; however, the germination percentage of the transgenic T1 seeds varied depending on the concentrations of N-AcPt. For example, at 0.2 mg ml^{-1} of N-AcPt, 89.9% of the seeds germinated while only 12.5% germinated in 1.0 mg ml^{-1} (data not shown).

The PCR data showed the effectiveness of the NS effect of N-AcPt on *argE* transgenic rice (Fig. 4C). *ArgE* and *hpt* transgenes were occasionally detected in plants that were germinated in $\leqslant$0.2 mg ml^{-1} of N-AcPt, while no transgenes were detected in those germinated in $\geqslant$0.5 mg ml^{-1}. This differential in the expression of the *argE* gene in the transgenic plants suggests that non-toxic N-AcPt, the substrate of *argE* deacetylase, was not sufficiently converted into the cytotoxic PPT in $\leqslant$0.2 mg ml^{-1}. However, sufficient conversion of N-AcPt into PPT was accomplished in the treatment of $\geqslant$0.5 mg ml^{-1} in the seed germination assay.

4. Discussion

PPT (glufosinate) (i.e., BA with two alanine residues removed), and N-AcPt are intermediate products in the BA pathway of soil-borne bacteria [24–26]. PPT was first isolated from *Streptomyces hygroscopicus* or *Streptomyces viridochromogenes* [27,28]. *Bar* and *pat* genes cloned from the bacteria confer resistant traits to Basta herbicide in transgenic plants by converting cytotoxic PPT into non-toxic N-AcPt [29,30]. In plant transformation, PPT has been widely used as PSA since it is the main ingredient of Basta herbicide and is toxic to plant cells by inhibiting the glutamine synthase, the key enzyme for ammonium assimilation in plants, leading to the depletion of glutamine, the rapid accumulation of harmful ammonia, and finally plant cell death; however, this does not occur with N-AcPt [4,31,32].

Otherwise, the *argE* gene encodes NAO deacetylase, which has an important role for cell proliferation because ornithine is required for the arginine and polyamine synthesis involved in DNA replication and cell division [33]. Furthermore, because of its broad substrate specificity [19], this enzyme could react with

[2] For interpretation of color in Fig. 3, the reader is referred to the web version of this article.

[3] For interpretation of color in Fig. 4, the reader is referred to the web version of this article.

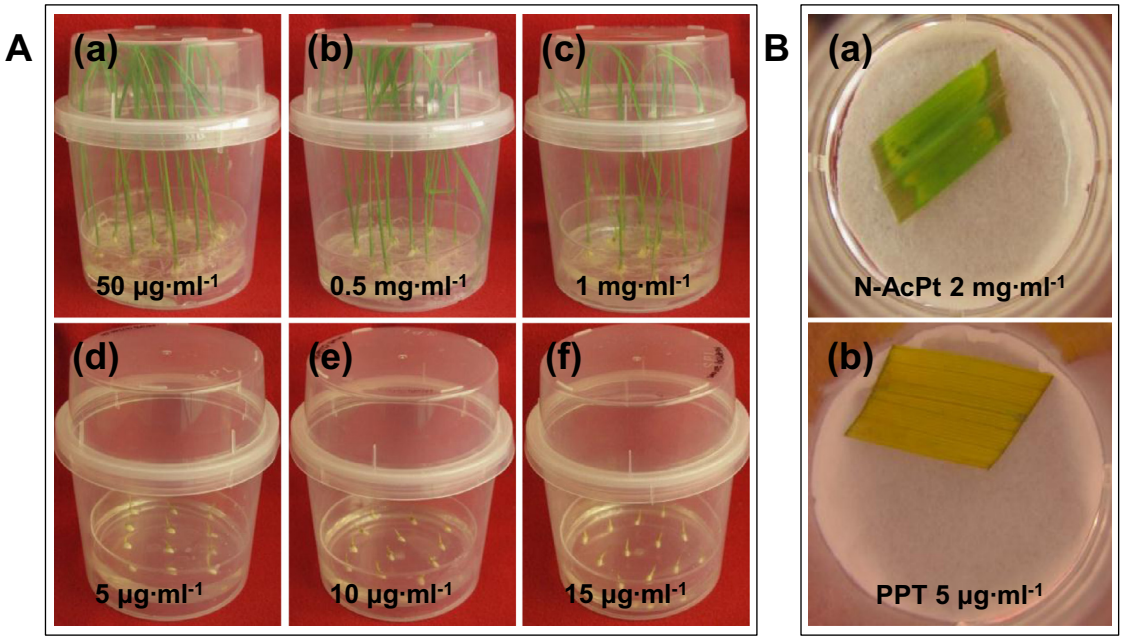

Fig. 3. Cytotoxicity test of N-AcPt and DL-PPT using WT rice plants. (A) Seed germination on hormone free MS basal medium containing various concentrations of N-AcPt (a–c) and DL-PPT (d–f) for 12 d. (B) Leaf discssoaked in 2 mg ml^{-1} N-AcPt and 5 μg ml^{-1} DL-PPT solutions for 5 d.

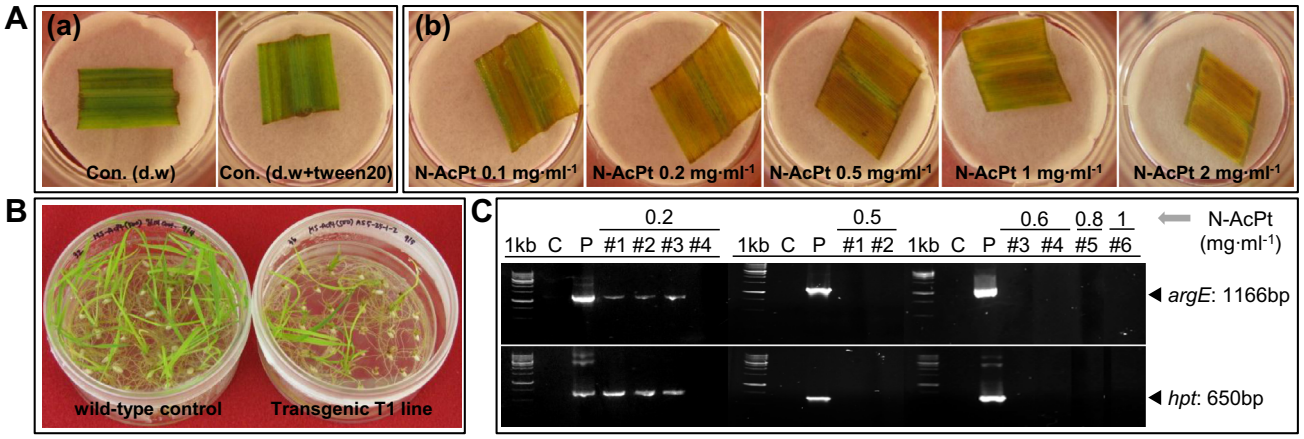

Fig. 4. Negative selection of *argE* transgenic rice using N-AcPt. (A) Leaf discs of *argE–hpt* transgenic T1 plants treated with different concentrations of N-AcPt. (a) Transgenic controls treated in distilled water with or without 0.1% tween 20, (b) negative selection effect of N-AcPt on leaf discs of T1 plants. (B) Germination of WT and transgenic T1 seeds on MS basal medium supplemented with 0.5 mg ml^{-1} N-AcPt. (C) Confirmation of negative selection effect of N-AcPt by PCR analysis with T1 plants; that were germinated under different concentrations of N-AcPt (0.2–1 mg ml^{-1}) but were severely restricted in growth.

non-toxic N-AcPt and convert it into cytotoxic PPT, thereby inducing self-destruction of the plant cell [14].

The transition between non-toxic N-AcPt and cytotoxic PPT could be selectively induced by the expression of the transgenes (*argE*, *bar* and/or *pat*), depending on the purpose of transformation. Our results demonstrated the possibilities of using *argE* gene and N-AcPt for NS in plant transformation. Based on the knowledge of *argE* gene and BA pathway, Kriete et al. developed a male sterility system involving the conversion of N-AcPt into PPT through the expression of tapetum-specific *argE* deacetylase from *Escherichia coli* [14]. However, this method is limited in use due to the small amount of N-AcPt produced by mutant *S. hygroscopicus* and the inconvenience of purifying N-AcPt from the media.

In our laboratory, N-AcPt was efficiently prepared from commercial DL-PPT by acetylation using acetic acid (AcOH) (and later THF was used) as a solvent and Ac_2O as a reactant. Although a minor application, THF is considered a relatively non-toxic solvent and often used as a solvent for polymerization. It can also be easily removed from the reaction mixture under reduced pressure because of its lower boiling point (66 °C) compared to that of AcOH (118–119 °C). NMR and ATR-FTIR data showed clear evidence of successful acetylation of PPT with THF and Ac_2O, thereby converting PPT into N-AcPt.

After application of N-AcPt, both WT and *argE* transgenic plants were differently influenced by treating the acetylated products prepared with different solvents. N-AcPt produced with AcOH occasionally caused cell destruction on the leaf discs of the non-transgenic plants (data not shown). This was unlikely to be due to the toxicity of N-AcPt itself, but to the remaining L-PPT or solvent not yet reacted [21]. N-AcPt prepared with THF and Ac_2O did not affect the germination of WT rice plants or show visual damage to leaf discs. Therefore, there is no harmful effect on plants caused by solvents and/or PPT that may remain after the acetylation reaction. Kriete et al. also reported that N-AcPt obtained from

S. hygroscopicus did not show harmful effects on various plants treated with 200 mg ml^{-1} and could remain in plant tissue for 10 mo with no metabolic disturbances. However, in *argE* transgenic tobacco, nontoxic N-AcPt was converted into toxic PPT and the plant tissue became damaged. At this point, a sufficient amount of N-AcPt (3 mg ml^{-1}) was needed to induce the self-destruction of the transgenic cells or tissues within several days after treatment [14].

With *argE–hpt* transgenic rice, 0.1 mg ml^{-1} of N-AcPt was sufficient to induce NS in the leaf assay. Also, a more rapid NS effect was observed in leaf discs than in percentage seed germination at relatively lower concentrations of N-AcPt (compare Figs. 3 and 4). The low concentration of N-AcPt ($\leqslant$0.2 mg ml^{-1}) in the medium did not induce the NS effect on *argE* transgenic seed. In PCR analysis, the *argE* transgenic plants survived on the medium containing $\leqslant$0.2 mg ml^{-1} of N-AcPt, yet both *hpt* and *argE* transgenes were frequently detected (Fig. 4). However, plant survival was reduced markedly at increased concentrations of N-AcPt. In $\geqslant$0.5 mg ml^{-1} of N-AcPt, no *argE* transgenic rice plants survived because sufficient amounts of PPT were accumulated by the expression of the *argE* gene within 2 wk.

In conclusion, we have shown the value on NS for desired transgene expression in *argE* transgenic rice. The advantages include easier preparation of N-AcPt using organic acetylation and rapid detection of negative selection effects. While the leaf disc assay was effective, the seed assay appears to be better adapted for mass production since the surviving plants are transgenic plants with the desired trait for direct use. Selection was best if $\geqslant$0.5 mg ml^{-1} of N-AcPt was treated in *argE* transgenic rice. We are currently using the proposed method to develop transgenic rice that resists environmental stress and is free of the selectable marker.

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