

A Novel Light-dependent Selection Marker System in Plants

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Introduction

Agricultural biotechnology has been continuously developing since the first transgenic plants were made in 1983. Recombinant DNA and plant transformation technologies, which introduce foreign genes into plants, have led to the advancement of molecular farming and improved agricultural production and economy.

Transgenic plants are easily generated by several available selection marker systems. Thus far, more than 50 selectable marker genes have been isolated from various sources^{1,2,3}. The three major groups of selection markers currently used for generating transgenic plants confer resistance to antibiotics, herbicides, or metabolic inhibitors¹. Among these, the four most widely used selection systems include: (i) kanamycin (Km) and neomycin phosphotransferase II (*nptII*); (ii) hygromycin B (Hyg) and hygromycin phosphotransferase (*hpt*); (iii) glufosinate and phosphinothricin N-acetyltransferase; and (iv) glyphosate and 5-enolpyruvylshikimate-3-phosphate synthase³. Although many selection systems have been reported, *nptII* was used most frequently in the United States to produce transgenic crop plants (soybean, cotton, canola, and corn) in 2001–2002³. Genes of 5-enolpyruvylshikimate-3-phosphate synthase and phosphinothricin N-acetyltransferase were used one-half and one-third as frequently as *nptII*, respectively³.

Although these antibiotic and herbicide resistance selection systems are useful in research and commercial applications, use of toxic selection agents in the field and expression of selection marker genes in transgenic plants have created public concern⁴. Koh et al. recently developed a light-dependent selection marker system that is a combination of a photosensitizer and its degrading enzyme. The system is a good candidate as a next generation selection marker system in that it does not target any enzymatic process in the plant and acts in a nonselective manner. Such a unique mode of action and a nonselective manner could help alleviate public concerns and boost the development of diverse genetically engineered crops.

Toxoflavin

Photosensitizers are commonly found as diverse forms (flavin, chlorophyll, coumarins, porphyrins, and cercosporin) in nature. Chlorophyll and porphyrins absorb photons and transduce light energy in photosynthetic organisms^{5,6}. Superoxide and H₂O₂ are produced during this process, but low levels of these reactive oxygen species can be quenched by carotenoids in plant cells. However, endogenous chlorophylls might damage plant cells under carbon dioxide deprivation, and exposure to eosin can inhibit photosynthesis⁵. Common dyes such as acridine orange, eosin Y, methylene blue, and safranin are also photosensitizers.

Toxoflavin was identified in 1934 as one of the phytotoxins produced by *Pseudomonas cocovenenans*. It produces superoxide (O₂⁻) and H₂O₂ during autorecycling oxidation under oxygen and light^{7,8}. We reported previously that *Burkholderia glumae* produces toxoflavin as a major virulence factor causing bacterial rice grain rot and demonstrated that toxoflavin biosynthesis in *B. glumae* is regulated by quorum sensing⁹. Like toxoflavin, the plant pathogen cercosporin, a light-activated perylenequinone toxin of fungal genus *Cercospora*, is reported as a photosensitizer^{6,10}. Cercosporin-generated active oxygen species cause peroxidation of lipids in plant cell membranes^{11,12}. Given that the light-dependent generation of superoxide and H₂O₂ by toxoflavin results in damage to plant cells, we hypothesized that the detoxification of toxoflavin would confer positive selection in the generation of transgenic plants.

TfIA

To identify a gene responsible for toxoflavin degradation, various environmental samples, such as soil, plant debris, and rice seeds, were tested to isolate bacteria that survive in toxoflavin solution. A bacterium was isolated from healthy rice seeds and identified as *Paenibacillus polymyxa*. The screening of a genomic library derived from *P. polymyxa* in *E. coli* HB101 found the fragment carrying a gene responsible for toxoflavin degradation. The gene *tflA* is 666 bp and encodes a protein, TfIA, expected to be 24.56 kDa, based on amino acid sequence. A comparison of TfIA amino acid sequences with the six previously reported members of the dioxygenase

superfamily exhibited 36.52% identity to a predicted ring-cleavage extradiol dioxygenase of *Exiguobacterium* sp. 255-15.

The optimum conditions for TflA are neutral pH and room temperature. Biochemical characterization of TflA revealed that Mn²⁺ and dithiothreitol, unlike other extradiol dioxygenase, were required for toxoflavin degradation. Substrate specificity of TflA was also examined with various toxoflavin derivatives: Toxoflavin, 3-methyltoxoflavin, 4,8-dihydrotoxoflavin, and 3-methylreumycin were degraded completely; reumycin and 3-methyl 4,8-dihydrotoxoflavin were slightly degraded; whereas fervenulin, 3-phenyltoxoflavin, 3-phenylreumycin, and 5-deazaflavin were not degraded by TflA. Therefore, TflA failed to degrade the toxoflavin derivatives with a phenyl group on the 3rd carbon, suggesting a substrate specificity of TflA.

Toxoflavin / tflA selection marker system

Because a single gene mediates toxoflavin detoxification, a combination of toxoflavin and the *tflA* gene would be a good candidate to use as a positive selection system for plant transformation. The phytotoxicity of toxoflavin is effective on broad ranges of monocots and dicots (**Fig. 1**). Because toxoflavin is a known photosensitizer, it is not surprising that all plant species in this study were sensitive to toxoflavin when exposed to light. Based on our results, we predict that all photosynthetic organisms are likely sensitive to various concentrations of toxoflavin.

For rice transformation in general, the Hyg / *hpt* selection system is preferred over Km and geneticin selection systems, because of its higher transformation frequency¹³.

We believe our novel toxoflavin / *tflA* selection system is a good candidate for rice transformation when additional markers are required, and further suggest two reasons our novel system could replace the current selection systems in rice (**Fig. 2**). First, the toxoflavin / *tflA* selection system is comparable to the currently used Hyg / *hpt* selection system. Second, in contrast with the Hyg / *hpt* selection, we did not observe any negative effects on calli proliferation and regeneration of rice grown on medium infused with toxoflavin. However, escapes through the callus selection and plant regeneration stages were frequent.

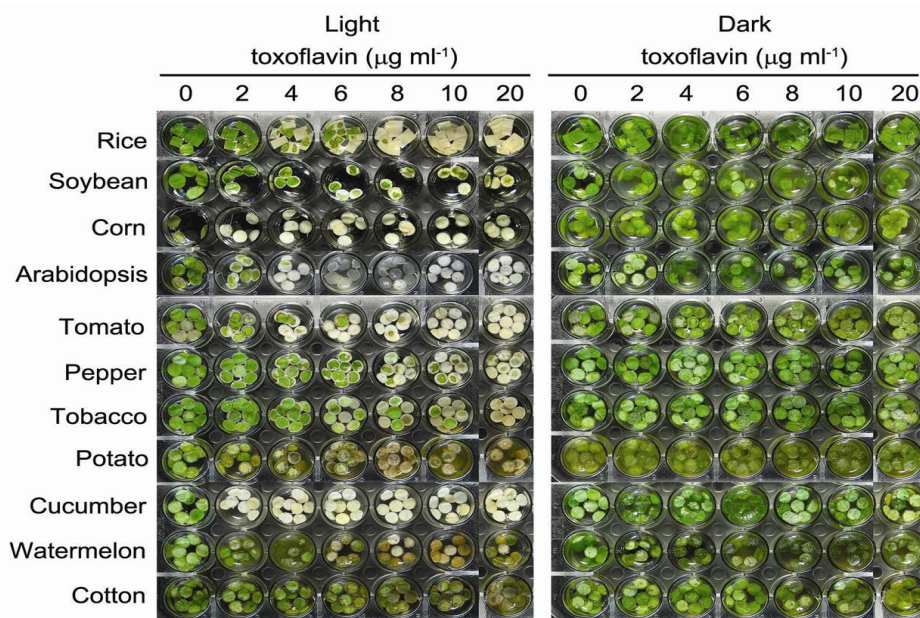


Figure 1. Sensitivity of plants to toxoflavin. Leaf discs of 12 different plants (rice, barley, corn, Arabidopsis, hot pepper, soybean, tomato, tobacco, cucumber, watermelon, melon, and zucchini) were immersed in toxoflavin (0–20 mg/L), incubated under light (124.5 µmol) or in the dark and photographed after 44 h. All 12 plant species were sensitive to toxoflavin only in the presence of light.²

We frequently have encountered false-positive selections from the Km/*nptII* selection system or the Hyg/*hpt* selection system in high-density screening of T1 transgenic Arabidopsis seeds. This may be because we select the high density transgenic seedlings from among all germinated seedlings 5 – 7 days after germination. With the toxoflavin / *tflA* selection system, we observed the clear separation of putative germinated transgenic plants from untransformed wild-type plants on the medium supplemented with toxoflavin compared to germinated plants grown on medium containing Hyg. Toxoflavin inhibited germination of the wild-type Arabidopsis seeds, eliminating false-positive selection of transgenic plants. Although root growth of transgenic Arabidopsis was retarded by toxoflavin, transfer to fresh culture media resulted in complete root growth recovery. Rooted transgenic Arabidopsis successfully flowered and showed no negative effects on plant growth and phenotype. Therefore, we

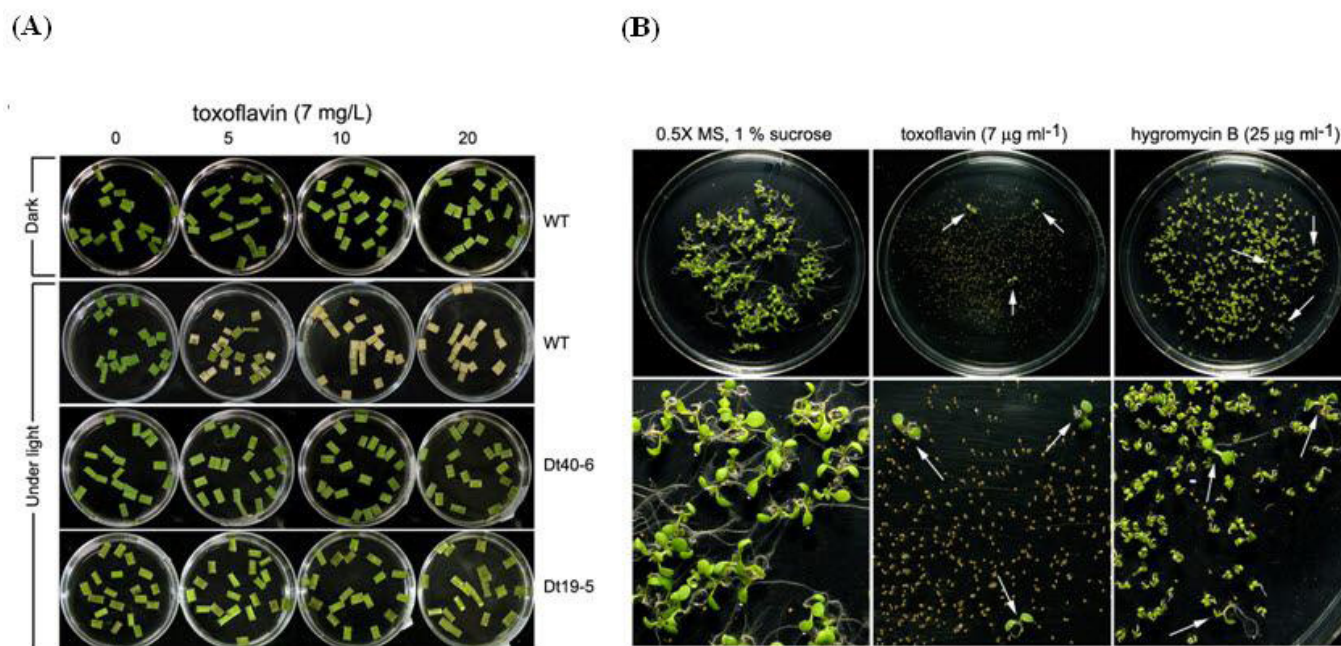


Figure 2. Transgenic rice and *Arabidopsis* containing the toxoflavin/*tfA* selection system. (a) Leaf disc assay of wild-type Dongjinbyeo and T4 transgenic Dongjinbyeo expressing *tfA* (Dt19-5 and Dt40-6). Leaf discs were immersed in toxoflavin (0–20 mg/L), incubated either under light (124.5 μmol) or in the dark and photographed after 40 h. (b) Hygromycin- or toxoflavin-resistant transgenic T1 *Arabidopsis* seedlings (arrows) were selected on 0.5 • MS with 1% sucrose medium containing no selection agent (top left), 7 mg/L toxoflavin (top middle), or 25 mg/L hygromycin B (top right). Bottom panels are close-up images of the top panels. Photos were taken 7 days after germination.

conclude there are no negative effects associated with the toxoflavin/*tfA* selection system in plant development. In addition to rice and *Arabidopsis*, the toxoflavin/*tfA* selection system is also reliable for other crop plants, such as tobacco, potato, pumpkin, and pepper, subjected to the transformation, and has comparative advantages in terms of being a flexible transformation method and a simple way to eliminate false-positive backgrounds.

Future perspectives

The use of naturally occurring photosensitizers such as toxoflavin as selection agents appears to give rapid and unambiguous selection results due to their unique phytotoxic mode of action. As a non-antibiotic or non-herbicidal selection system, the toxoflavin/*tfA* selection system is a new and alternative comprehensive selection marker system that can be applied to many plant species. When versatile selection markers are needed for gene stacking, the toxoflavin/*tfA* selection system is a good candidate, with a unique mode of action compared to the currently available selection marker systems. In particular, the toxoflavin/*tfA* selection system might be useful for generating transgenic plants where high false positive backgrounds are problematic, as with the current selection marker systems.

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