Note
Toxoflavin Lyase Enzyme as a Marker for Selecting Potato Plant Transformants

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This study established a new system for potato transformation using toxoflavin as selection agent and toxoflavin lyase (tflA) as selectable marker gene. Potato plants expressing tflA was successfully transformed on toxoflavin medium with 27% efficiency, similar to that for the hygromycin/hpt selection system. The transgenic potato expressing tflA also showed resistance to Burkholderia glumea infection.

Key words: toxoflavin lyase (tflA); selection marker system; light-dependence; transformation efficiency

Although marker-free techniques are available, selectable marker genes (SMGs), including the neomycin phosphotransferase II gene (nptII), the hygromycin phosphotransferase II gene (hptII), and the bialaphos resistance gene (bar), are useful in obtaining transgenic potato plants efficiently.¹,² Most SMGs are derived from microorganisms and confer antibiotic or herbicide resistance that may pose risks to humans or the environment.³ Such concerns as to transgenic plants can be alleviated by the use of the current generation of marker-free transgenic plants, or of antibiotic- and herbicide-independent, SMGs of plant origin. The principle behind the development of these less controversial, non-antibiotic marker systems is that the gene product of a selectable marker converts the selection agent into a compound that has positive effects on the transformed cells. In 1934, toxoflavin was identified as a phytotoxin produced by Pseudomonas cocovenenans, and was thought to produce superoxide and H₂O₂ under conditions of oxygen and light.⁴,⁵ Given that light-dependent generation of superoxide and H₂O₂ by toxoflavin results in damage to plant cells, Koh et al.⁶ suggested that detoxification of toxoflavin would make possible positive selection in the generation of transgenic plants. Based on those experiments, the toxoflavin/tflA transformation system was reported first for Arabidopsis.⁶⁰

Present study was conducted to construct a new binary vector using this selection system for potato transformation.

The sensitivity of potato leaves (Solanum tuberosum L. cv. Desiree) to toxoflavin (PKF118-310, Sigma-Aldrich, St. Louis, MO) was tested. No effect was observed in potato leaves subjected to various toxoflavin concentrations (4 to 40 mg/L) in the dark, but, potato leaves were sensitive to all concentrations of toxoflavin within 2 d under visible light (145 μmol photons·m⁻²·s⁻¹) conditions (Fig. 1A). Furthermore, we applied different intensities of light (6.5 and 145 μmol photons·m⁻²·s⁻¹) to determine the phytotoxic effects of toxoflavin on shoot regeneration. At lower intensities, regeneration rates were similar as between toxoflavin treatment and no treatment. However, at an intensity of 145 μmol photons, shoot regeneration decreased sharply, to 35% and to 20%, in the samples treated with toxoflavin (Fig. 1B). In order to determine the proper toxoflavin concentration to create selection pressure, we cultured potato leaf discs at various toxoflavin concentrations (0, 0.1, 0.2, 1, 2, 5, and 40 mg/L). At high concentrations, 5–40 mg/L of toxoflavin, leaf segments browned without callus formation (Fig. 1C). On the other hand, following exposure to 0.1–2 mg/L of toxoflavin, callus was induced at the edge of the leaf discs after 10 d, and 4 weeks later, shoots appeared from the callus at 40–70% (Fig. 1D). A total of 100 explants were plated equally in three replication of the experiment at each concentration. The optimum concentration of toxoflavin was determined to be 5 mg/L to produce selection pressure for potato transformation. Full-length tflA was amplified from template pJ9046 by PCR (95°C for 5 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, and 72°C for 10 min) using forward primer tflA-F (5’-cctgagactcctgattaaa-caggcttac-3’) and reverse primer tflA-R (5’-ctcgagatgacttcgattaaa-caggcttac-3’). After XhoI digestion, the PCR product was introduced into pCAMBIA1304 (Cambia, Canberra, Australia), and the hptII gene was replaced with tflA, resulting in p1304-tflA for potato transformation (Fig. 1E). The hptII gene was also replaced with the XhoI 0.85 kb fragment of the nptII gene digested from pCAMBIA2301, resulting in p1304-npt, to compare the transformation efficiency of the selectable marker. Potato leaf discs were inoculated with A. tumefaciens EHA105 harboring binary vector p1304-tflA, p1304-npt, or p1304. About 1 month later, the leaf discs produced a number of shoots on a selection medium containing 5 mg/L of toxoflavin for tflA, 100 mg/L of kanamycin

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Abbreviations: Bar, bialaphos; HptII, hygromycin phosphotransferase II; NptII, neomycin phosphotransferase II; SMGs, selectable marker genes; tflA, toxoflavin lyase
for *nptII*, and 4 mg/L of hygromycin for *hptII*. All rooted plants in the hormone-free MS medium were subjected to molecular analysis.

The Southern blot analysis results for representative samples of the *tflA*-positive potato confirmed stable integration of the *tflA* gene in the transgenic plants with a variable integration pattern, while no hybridization signal was observed in the wild-type control potato plants (Fig. 2A). The mRNA transcripts of *tflA* and GUS were also expressed with similar patterns (Fig. 2B and C, respectively). The leaf discs of these lines exhibited toxoflavin resistance after 3 d when immersed in 4 mg/L of toxoflavin under light, while the leaf discs of the wild-type control and independent line 7 were bleached (Fig. 2E). The transgenic potato plants expressing *tflA* showed resistance to *Burkholderia glumae* infection. Detached leaves were infected with 10 μL of diluted *B. glumae* cell solution (1 × 10⁵ cfu/plant) in the central area of the leaf after a small wound was made with a toothpick. Wild-type control leaves showed severe necrosis, but the leaves of transgenic line 3 showed no symptoms following *B. glumae* infection (Fig. 2F). These results indicate that *tflA* expression can be used not only as a selection marker but also to induce resistance to *B. glumae*. Similarly, Zhang *et al.* have reported that salt-tolerance gene *rstB*, used as a selectable marker gene in tobacco, resulted in improved salt tolerance.

Transformation frequency was calculated as the percentage of explants giving rise to PCR and Southern-positive plants from the total number of co-cultivated explants.
vated explants per experiment. The highest number of regenerated shoots (63 ± 8.0) was obtained with 35S::npt (Table 1). The other expression vectors, 35S::hpt and 35S::tflA, showed similar numbers of regenerated shoots (36.7 ± 4.16 and 35 ± 5.0, respectively). Based on the results of PCR and Southern blot, the average transformation frequencies of nptII, hptII, and tflAwere calculated to be 36%, 27.7%, and 27% respectively. Previous studies have demonstrated the utility of new genes as efficient selectable markers in tobacco, with frequencies of about 20%. \(^5\) When using nptII, false-positive selections are frequently encountered from multiple shoot regeneration on the calli. Some plant tissue is not readily killed even by high levels of kanamycin. \(^6\) Transgenic potato plants selected by the toxoflavin/tflA system could be distinguished unambiguously from false transformants during rooting, as with the hygromycin/hptII selection system. Koh et al. \(^7\) have reported that selection was visually more distinct using the toxoflavin/tflA system in Arabidopsis. Having a normal phenotype without adverse effects is also a critical factor in choosing a selectable marker. The toxoflavin/tflA selection system in the potato caused no abnormalities in plant growth or development, either in vitro or in field cultivation (data not shown).

In sum, tflA was found to be a useful alternative selection marker that is suitable for use in the potato plant where high false-positive backgrounds are problematic. The present study is the first to confirm use of the tflA gene as a non-antibiotic selectable marker that is useful as a single selectable marker for potato transformation.

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**References**