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Short Communication

Fluorometric Analysis of Transient Gene Expression in Broccoli Seedlings

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The genus *Brassica* includes many crop species that are important for the production of vegetable and oil. *B. oleracea, B. rapa, B. napus, B. juncea, B. campestris, B. nigra* are examples. So far, the necessary genetic variability in these economic species has been created through genetic approach by introducing novel genes or altering the expression of existing genes. To establish new cultivars for higher resistance to pathogens and herbicides and for better quality of oil and nutrition, their transgenesis is now a routine practice owing to *Agrobacterium*-mediated transformation.[1-4] *B. oleracea* L. var. italica, commonly called as broccoli, is broadly cultivated for vegetable use. Its seedling, broccoli sprout, is consumed as a healthy food also. It can be prepared from seed within a week or so.

The generation of stably transformed transgenic plants is the most critical step towards plant-based molecular farming. This procedure, however, requires considerable investment in time and labor. Recently, transient expression system has been alternatively proposed due to its several advantages including simplicity and rapidity.[5,6] Expressed proteins can be extracted from leaf tissues within a week following agroinfiltation process.[7] Meanwhile, transformed seedling tissues have been little utilized for this purpose.

Under favorable conditions, the seed of a higher plant develops towards a seedling through the stages of seed imbibition, radicle emergence and subsequent seedling growth. During this progression, seedling may attain a few orders of magnitudes greater in size than the seed embryo. It is true that many of vegetable seedlings can be harvested soon after sowing. Therefore, it may be valuable to develop the seedling as a rapid transient expression system if efficient genetic transformation method is provided.

In this work, broccoli seedling-based gene expression was evaluated following chemical wounding-assisted agroinfiltration. It was quantitatively analyzed by fluorometric GUS assay. As chemical wounding agents, hydrogen peroxide (HPX) and sodium hydrosulfite (SHS) solution were used. They are common in use as a bleaching agent due to a strong oxidizing property of HPX and to a reducing power of SHS in solution.

Broccoli seeds that were sold for growing vegetable sprout at home were surface-sterilized with 0.4% sodium hypochlorite solution for 1 min, washed with sterile water and then soaked with sterile water for 24 h at 4°C for imbibition. Seeds were placed onto pre-wet paper towel for germination and seedling growth in the dark under humid condition at 25°C. Next day, young seedling (1 day-old seedling: D1S) could be observed in most experiments. Agroinfiltration was carried out separately for broccoli seedlings of D1S, D2S (2 day-old seedling), and D3S (3 day-old seedling). For GUS reporter gene transformation, the seedlings in 100 m*l* of sterilized 1/2 × MS medium were vacuum-infiltrated for 10 min with 500 μ*l* of 16 h-grown *Agrobacterium* cell cultures carrying pBI121 GUS expression vector. The agroinfiltrated seedlings were briefly blotted on paper towel to remove excess of liquid and then air-dried in the laminar flow bench for 30 min. The seedlings then were transferred onto pre-wet paper towel at 27°C in the dark for cocultivation and further seedling growth. From sowing to seedling harvest, 8 days were given in total. On day 8, the transformed seedlings were analyzed for GUS gene expression. For chemical wounding-assisted genetic transformation, seedlings (D1S, D2S and D3S) were treated with 3-7% HPX or 1-2% SHS for 3 min, thoroughly washed with distilled water and then air-dried in the laminar flow bench for 30 min prior to agroinfiltation. GUS enzyme activity was determined according to the method by Jefferson.[8] The transformed broccoli seedlings were sterilized using 0.4% sodium hypochlorite solution for 3 min and then washed several times with distilled water. The seedlings were extracted with cold GUS extraction buffer (50 mM NaPO4 [pH 7.0], 10 mM EDTA, 0.1% sarkosyl, 0.1% Triton X-100, and 10 mM DTT). After centrifugation (14,000 rpm, 5 min, 4°C) using a minicentrifuge, 10 μ*l* of the supernatant was mixed with 90 μ*l* of assay buffer (extraction buffer containing 1 mM 4-methylumbelliferyl β-D-glucuronide [4-MUG]) for 3 h at 37°C and then added with 200 μ*l* of 0.2 M Na_2CO_3 to stop the reaction. The 4-MUG fluorescence product, 4-MU (4-methylumbelliferone), was measured on a PerkinElmer VIVTOR 3 microtiterplate reader. Total protein content was determined using Bio-Rad protein assay kit. Total RNAs that were isolated from the seedlings using Tri-reagent (MRC Inc.) were reversely transcribed using M-MuLV reverse transcriptase and oligo-dT_{15} primer for 1 h at 42°C. Synthetic primers (5'-cattacagtctggatcgcgaa-3', the forward strand; 5'-aagttcatgccagtccagcg-3' for the reverse) that were designed to amplify 1.7 kb DNA from 1.8 kb GUS gene were used for 30 cycles of PCR (94°C, 45 s/52°C, 45 s/72°C, 90 s).

Fig. 1 shows the level of GUS enzyme activity in transformed broccoli seedlings determined by fluorometric assay. Non-

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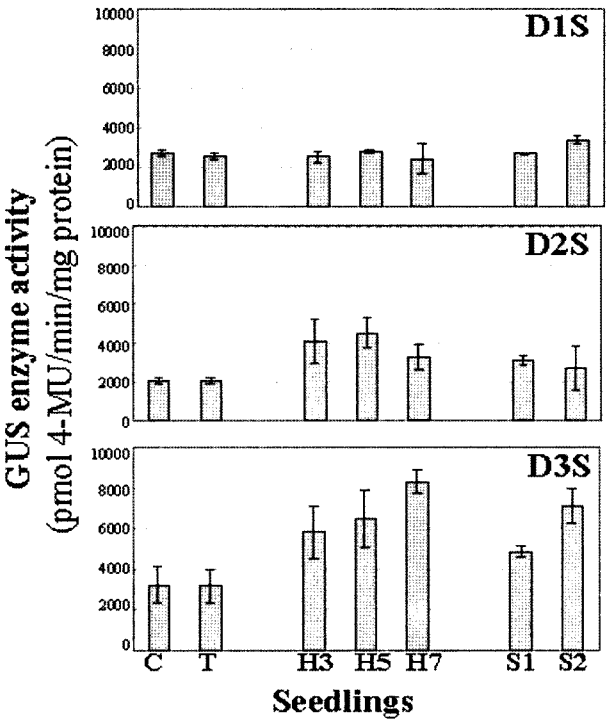

Fig. 1. Fluorometric GUS activity assay in transformed broccoli seedlings. *Agrobacterium*-mediated transformation via vacuum-infiltration was performed to broccoli seedlings at the age of 1, 2 and 3 day (D1S, D2S, D3S) following germination. Non-transformed (C), transformed (T), HPX-treated and then transformed (H3, H5, H7) and SHS-treated and then transformed (S1 and S2) seedlings were analyzed by fluorometric GUS activity assay. Numbers attached to H and S represent the treated concentration (%) of HPX and SHS, respectively.

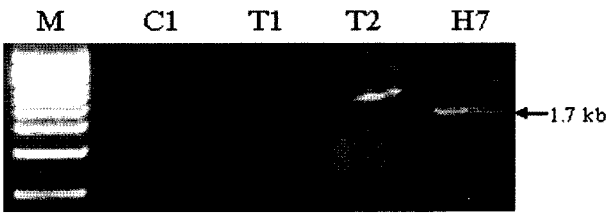

Fig. 2. RT-PCR analysis of GUS mRNA expression in transformed broccoli seedlings. RT-PCR was performed for non-transformed (C), transformed D2S (T1). transformed D3S (T2) and 7% HPX-treated and then transformed (H7) seedlings.

transformed broccoli seedlings as a control (C) and transformed seedlings (T) similarly showed the GUS activity to each other, implying that the common agroinfiltration process was not efficiently working for the transformation of broccoli seedlings, D1S, D2S and D3S under our experimental conditions. D1S, D2S and D3S were treated with HPX and SHS prior to agroinfiltation. Generally, broccoli seedling growth was retarded more as the chemical treatment became stronger and longer, simply reflecting their toxicity to seedling tissues as a bleaching agent. In this work, the treatment was adjusted to 3-7% for HPX and 1-2% for SHS for 3 min duration. Under these conditions, broccoli seedling growth seemed to be slightly but not severely harmed when in comparison to the control growth.

It was shown that chemical wounding to D1S tissues seemed overall ineffective, even though 2% SHS treatment to D1S resulted in somewhat higher level of the GUS activity. This probably indicated that broccoli seedlings at younger age like D1S was more calcitrant to *Agrobacterium* infection regardless of chemical wound generation. In contrast, D2S and D3S with the chemical wounds seemed to become more feasible for the *Agrobacterium* infection by demonstrating significantly higher level of the GUS enzyme activity. For competent *Agrobacterium*-mediated transformation, D3S was determined to be the most appropriate seedling tissue when treated with 7% HPX. It was estimated that 4-MU fluorescence products in H7-D3S had 3 fold increase compared to the background values in C or T. It was also notable that S2-D3S showed 2.5 fold increases. The increment of H7- and S2-D3S could be expected much higher if the noise value is minimized by using purified seedling extracts.

The results of fluorometric GUS analysis was further evaluated at molecular level. mRNAs that were isolated from the transformed seedlings were reversely transcribed for PCR amplification. Fig. 2 shows the results. No DNA product was detected from either nontransformed (C) or transformed D2S and D3S (T1, T2) seedlings. This probably explained the background level of 4-MU products in the transformed D2S and D3S. In contrast, 1.7 kb DNA was clearly detectable from D3S administered with 7% HPX (H7). This was well in correlation with the result from GUS enzyme assay.

Although physical wounding via sonication,[9] silicon carbide[10] and sand[11] have been reported to facilitate *Agrobacterium*-mediated genetic transformation, chemical wounding method was introduced for broccoli seedling transformation in this study. From the results of the analysis of GUS enzyme activity and mRNA expression, it was suggested that broccoli seedlings at the age of 3 day could be used as a host for the rapid production of recombinant proteins if they, prior to agroinfiltration, were properly treated with HPX or SHS. Older ones at the age of 4 or more might be suggested also. For 7 day scheme for protein production, however, D3S could be more ideal one if we consider the scheme of protein production within a week that includes seedling growth for 3 days and the following cocultivation and transformed cell increment for 4 days.

Besides broccoli seedling, it would be apparently reasonable for other seedling species to be considered as well. At this moment, it remains to be examined whether HPX and SHS are also applicable for others than broccoli seedling transformation. Edibility of broccoli seedlings as sprouts will be a desirable property for the production of edible vaccines in addition to recombinant pharmaceutical proteins of high value.

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