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Effect of Sodium Hydrosulfite Solution on *Agrobacterium*-Mediated Chinese Cabbage Transformation and Transient Expression

Hee-Sung Park*, Dong-Il Shin

Department of Biotechnology, Catholic University of Daegu, Kyungsan 712-702, Korea

Abstract

We investigated chemical-wounding effect on *Agrobacterium*-mediated Chinese cabbage transformation via vacuum infiltration. Pre-germinated or germinating Chinese cabbage seeds were infiltrated with *Agrobacterium tumefaciens* LBA4404 cells carrying either GUS gene (pBI121) or hepatitis B virus surface antigen DNA (pBIHBsAg). Prior to agroinfiltration process, the seeds were soaked in sodium hydrosulfite (SHS) solution or just in sterile water as a control. Comparative transformation efficiency was determined by both of histochemistry and ELISA. We could demonstrate that SHS solution treatment especially to 1-day or 2-days old germinating seeds efficiently improved transformation process, and therefore, transient expression level. This strongly indicated that *Agrobacterium* infection could be facilitated indeed by SHS-causing wounds on Chinese cabbage seeds.

Key words: Sodium hydrosulfite, Chemical wounding, Agroinfiltration, Transformation efficiency

Introduction

Plant transformation can be accomplished by the use of modern gene delivery techniques such as biolistic bombardment, *Agrobacterium*-mediated transformation, electroporation and viral infection (Doran 2000; Fisher et al. 1999; Giddings 2001). Although its performance has purposed for the construction of the stable transgenic plant lines, it, recently, has aimed at foreign gene expression in a transient mode, so called, transient gene expression. For transient expression. the viral infection method for transient expression system employs recombinant viral vectors to infect plants and produce recombinant protein systemically by their replicated viral progeny throughout the whole plant body. Also, *Agrobacterium*-mediated transformation method can be performed by infiltrating (agroinfiltration) plant leaves or leaf discs with recombinant *Agrobacterium* cells for temporary target protein expression without selection procedure (Bechtold et al. 1993). To ameliorate the process of *Agrobacterium* infection, some assisting methodologies have been devised; that is, chemical supplementation such as acetosyringone (Lohrke et al. 2001) and physical wounding by sonication (Flores Solis et al. 2003). Due to advantages such as rapidity, simplicity and cost-effectiveness, transient expression system has been widely assessed in the efforts to produce recombinant proteins of high value (Wagner et al. 2004; Fisher et al. 2000; Joh et al. 2005).

Many of vegetable seeds can be easily germinated and grown to healthy seedlings in a simple device within a week. Seed/seedling system, therefore, may be worth of being developed as one of the transient expression systems because of its massive cell multiplication within a short period time with ease. In this study, Chinese cabbage (*Brassica rapa* ssp. pekinensis) was chosen and evaluated for its transient protein expression level in its seedlings following pregerminated- or germinating-seed transformation by agroinfiltration. Chinese cabbage belongs to the plant species with low transformation efficiency (Cho et al. 2001; Cho et al. 2003; Christey et al. 1999). We also tested chemical wounding-assisted *Agrobacterium*-mediated seed/seedling transformation (CAAT) because it could be expected as an alternative tool instead of physical wounding to increase *Agrobacterium* infection (Flores Solis et al. 2003). Sodium

* Corresponding author, E-mail: hspark@cu.ac.kr
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hydrosulfite ($Na_2S_2O_4$), a toxic chemical with a sulforous odor, was tested for CAAT due to its reducing power and abrasive properties when dissolved in water. Sodium hydrosulfite is generally used as one of chemical abrasives to kill microorganism. Transformation efficiency and transient expression level were analyzed by histochemical staining to detect β-glucronidase (GUS) expression and ELISA to determine HBsAg protein content, respectively.

Materials and Methods

Seeds, Germination, Transformation

Chinese cabbage seeds were purchased from a local company. Seeds were sterilized using a 0.4% (w/v) sodium hypochlorite solution for 1 min and thoroughly washed with sterile water. They were soaked in sterile water at 4°C for 24 hr. For germination, the imbibed seeds were placed in petri plate with pre-wet 2-3 layers of filter paper. At 27°C in the dark, seeds were cultivated for 6 days. Seeds at the day of planting (day-0-seed) or seeds in germination at following days (day-1-seed or day-2-seed) were independently agroinfiltrated. Seeds in sterile water were vacuum-infiltrated for 10 min with *Agrobacterium* tumefaciens LBA4404 harboring either pBI121 containing *gus* reporter gene (Clontech, USA) under the promoter of CaMV35S promoter or pBIHBsAg containing 0.7 kb HBsAg DNA placed in the site of *gus* DNA instead (details will be submitted elsewhere). Following agroinfiltration, seeds were blotted on paper tissue and then put back into petri plate for growth.

Chemical Wounding

Seeds were immersed in 1% or 2% sodium hydrosulfite solution and then vacuum-infiltrated for several minutes. Seeds were then washed thoroughly with distilled water prior to agroinfiltration.

Histochemical GUS Assay

Histochemical detection of GUS gene expression of 6-day cultivated sprouts was carried out as described by Jefferson (1987) using a slightly modified solution containing 1 mM X-gluc in 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM ferrocyanide and 5% polyvinylpyrrolidone-40 (Schaart et al. 2002).

ELISA

Sprouts were homogenized in extraction buffer containing 20 mM sodium phosphate (pH 7.0), 0.15 M NaCl, 20 mM sodium ascorbate, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1x proteinase inhibitor (Roche) (Joung et al. 2004). Cleared homogenate collected from two rounds of centrifugation (12,000 × g, 15 min) was analyzed for HBsAg protein content by ELISA using Abbott IMx detector system. Total soluble protein (TSP) was determined according to Bio-Rad Protein Assay system.

Results and Discussion

Effect of sodium hydrosulfite (SHS) solution on seed germination/growth

SHS toxicity was determined for the seeds at different ages. In aqueous solution containing from 1 to 3% SHS (w/v), day-0- (pre-germinated stage), 1- and 2-seeds (germinating period) were vacuum-infiltrated for 1 to 5 min at room temperature and then thoroughly washed with distilled to place them back to growth container. Their sprouts at the day of 6th were morphologically compared to the control sprouts mocked with water. Generally, 2-3% SHS solution exerted a harmful effect on normal seed growth if it continued for 4-5 min. By even a mild SHS treatment (1-2% solution for up to 4 min), seeds got still influenced by showing a delayed growth and stunted sprouts to some extent (data not shown). From this results, we set up an chemical wounding experiment by means of soaking seeds in 1 or 2% SHS solution for 2 or 4 min.

GUS activity in Chinese cabbage sprouts

GUS activity was investigated via histochemical staining of 6-days-old sprouts which were derived from seeds of day-0, 1, or 2 with or without SHS treatment prior to agroinfiltration. The results are shown in Fig. 1. In Fig. 1. A., both of non-transformed (Con) and the transformed sprouts (Trans) from non-SHS treated day-0-seed display very faint clues of GUS expression. Similarly, GUS-positive staining was not distinctively displayed from sprouts of transformed day-1- and day-2-seed (data not shown). Therefore, Chinese cabbage seeds seemed less liable to agroinfiltration-mediated transformation under our experimental condition. However, SHS treatment evidently worked in favor of *Agrobacterium* infiltration-mediated seed transformation. From almost all of the sprouts from SHS-treated seeds, GUS positive signs could be well detected as shown in Fig. 1. B. In regards of the seed age, day-1-seed seemed more suitable to SHS-assisted agroinfiltration than day-0- and day-2-seed. Prolonged SHS treatment (4 min instead of 2 min) worked seemingly

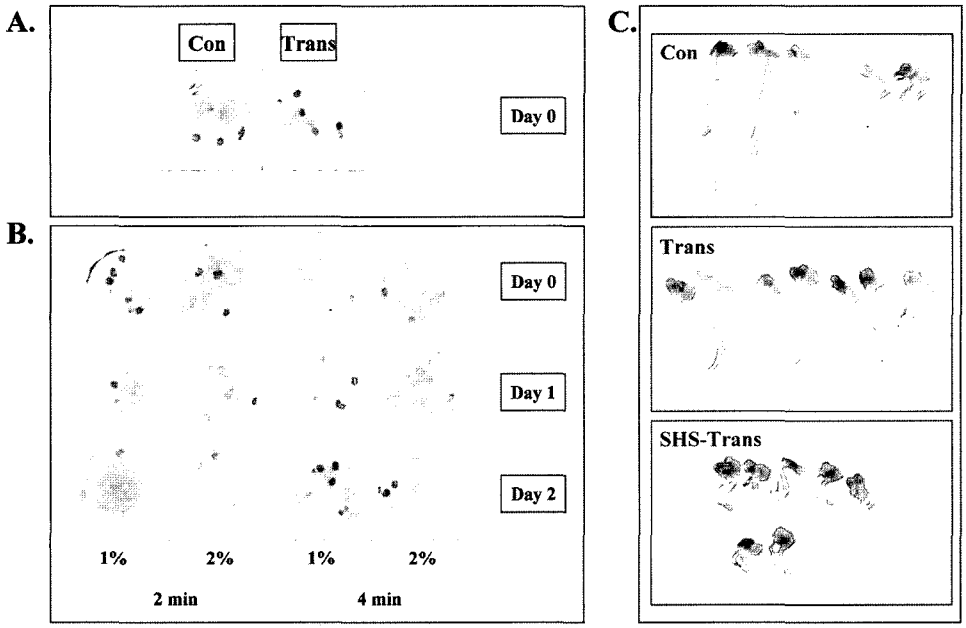

Figure 1. Histochemical detection of GUS expression in Chinese cabbage sprouts. A: Con; non-transformed sprouts. Trans; Sprouts of agroinfiltrated day-0-seed. B: Day-0-, 1-, and 2-seeds were treated with sodium hydrosulfite (1 or 2% for 2 or 4 min) and then agroinfiltrated. At the 6th day of cultivation, sprouts were analyzed for GUS expression. C: Closed look of GUS-stained sprouts. Con, Trans, and SHS-Trans are sprouts from non-transformed seeds, transformed seeds at day-0 and transformed seeds at day-1 with sodium hydrosulfite treated (2%, 4 min), respectively.

A.

Sprouts	TSP (μg/ml)	HBsAg (ng/ml)
Con	345.2	20.12
X-0-0-0	355.4	21.55
SS1-2-0	354.5	16.46
SS1-4-0	369.5	21.14
SS2-2-0	354.5	51.11
SS2-4-0	354.0	24.91
SS1-2-1	354.0	38.54
SS1-4-1	393.0	38.26
SS2-2-1	342.5	119.38
SS2-4-1	392.5	107.61
SS1-2-2	347.3	44.42
SS1-4-2	388.3	66.81
SS2-2-2	366.8	88.19
SS2-4-2	388.1	77.81

B.

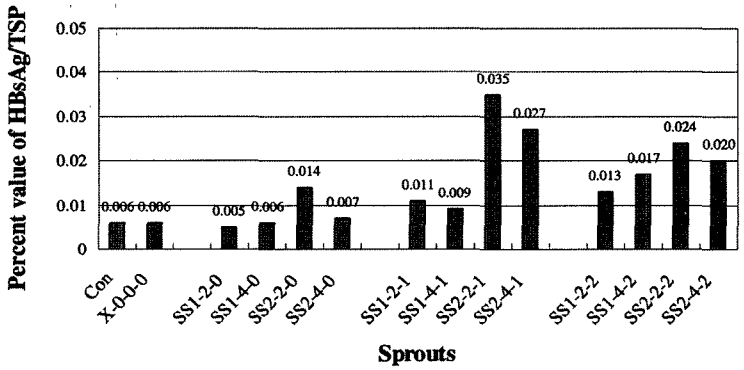

Figure 2. HBsAg protein content determined from transformed Chinese cabbage sprouts. A, B: Numbers attached for sprouts X (non-sodium hydrosulfite treated) or SS (sodium hydrosulfite treated) indicate, in the order, sodium hydrosulfite concentration (1 or 2%), treatment time (2 or 4 min) and seed age (0, 1, and 2) for agroinfiltration. B: Percent value of HBsAg protein contained in total soluble protein (TSP).

better in case of day-0-seed. For day-2-seed, shorter treatment (2 min instead of 4 min) improved to some extent. Meanwhile, either 1 or 2% of SHS solution similarly affected GUS transformation for seeds in ages of 0, 1, and 2. These results indicated that SHS treatment somehow assisted *Agrobacterium*-mediated transformation probably by generating wounds on seed surface and, therefore, facilitating the process. But, the efficiency differed according to seed ages (pre-

germinated and germinating stage) and treatment duration. In more detailed look of GUS stained sprouts (Fig. 1. C), SHS-Trans (sprouts from day-1-seed treated for 4 min with 2% SHS) showed relatively shorter growth but displayed strong GUS-positive signs from hypocotyl as well as cotyledon. However, this GUS-positivity was also observed from some selected sprouts of either Con or non-SHS Trans. This could probably mean to be false-positive (Fig. 1, C. Con) or false-negative (A. Trans) (Jefferson et al. 1987; Vitha et al. 1995). At this point, we determined to quantitate the efficiency of Chinese cabbage seed transformation by means of ELISA for HBsAg transgenic sprouts. HBsAg as a preventive vaccine antigen against HBV infection, has been tremendously investigated and well characterized (Joung et al. 2004; Sojikul et al. 2003; Sunil Kumar et al. 2003).

Quantitative estimation of Chinese cabbage seed transformation by ELISA

To estimate SHS efficiency for Chinese cabbage seed transformation, we carried out ELISA for HBsAg antigen determination instead of fluorometric assay for GUS enzyme using 4-methyllumbelliferyl β-D glucronide because of feasibility in our lab condition. HBsAg-transformed sprouts were cultivated and extracted for total soluble protein (TSP) preparation. In fact, SHS treatment and non-treatment was performed for GUS as described above and HBsAg transformation procedure under the same experimental condition. From TSP, HBsAg content was measured using Abbott IMx ELISA system together with rHBsAg vaccine antigen by Boryoung Biopharma (Korea) as a standard. The results are shown in Fig. 2, A. Fig. 2, B represents percent value of HBsAg protein contained in TSP. From X-0-0-0 (sprouts of day-0-seed with no SHS treatment), HBsAg protein was barely detected to show 0.006% value (see Fig. 3, B) which was equivalent to the value from non-transformed (Con) sprouts. This results indicated that GUS-stain from some of either Con or Trans sprouts (Fig. 1) meant nearly false-positive. So, Chinese cabbage seeds could be suggested for their limited nature of agroinfiltration-mediated transformation whether the seeds in pre-germinated or germinating stage. SHS treatment, however, remarkably changed the nature by showing HBsAg content up to 0.035% value (see SS2-2-1: 2% SHS for 2 min to day-1-seed). Estimation over 0.020% value could be obtained from SS2-4-1 (2%, 4 min, day-1-seed), SS2-2-2 (2%, 2 min, day-2-seed), and SS2-4-2 (2%, 4 min, day-2-seed). SHS-assisted transformation occurred mostly for day-1- and 2-seed but not for day-0-seed. These estimated HBsAg contents correlated very well with the results of histochemical detection of GUS expression especially in cases of day-1- and 2-seed transformation.

For the sole purpose of recombinant protein production in plant, transient expression system may be more promising than the stable transgenic plant lines in terms of time, simplicity and cost. From this point, transient expression system hiring seed/seedling could be the one that can be readily evaluated. Seedlings can be obtained shortly after planting the seeds under proper environment by the process of rapid and massive increases in total cell number and cell volume. In this study, Chinese cabbage seedlings were determined to show a greatly improved HBsAg protein synthesis by SHS treatment to germinating seeds prior to agroinfiltration, implying that wounds presumably caused by SHS might enhance *Agrobacterium* infection. Although Chinese cabbage seedlings via CAAT demonstrated a possibility of rapid protein production, further evaluation of seed/seedling species, chemical abrasives and CAAT condition will be required to establish a more efficient seed/seedling system for transient protein expression.

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