Hydrogen Peroxide Effect on *Agrobacterium*-Mediated Alfalfa Sprouts Transformation

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Alfalfa (*Medicago sativa* L.) is a valuable source of protein for livestock industries as well as of sprout vegetable for human consumption. So far, the necessary genetic variability in alfalfa has been created through genetic approach by introducing novel genes or altering the expression of existing genes. Alfalfa transgenesis is now a routine practice owing to *Agrobacterium*-mediated transformation.[1,2,3] In this study, pre-germinated or germinating alfalfa seeds were tested for their *Agrobacterium*-mediated transformation via vacuum-infiltration (agroinfiltration). Transformation efficiencies of conventional and hydrogen peroxide (HX)-treated agroinfiltration were compared through histochemical detection of GUS (β-glucuronidase) gene expression and heterologous hepatitis B virus surface antigen (HBsAg) synthesis; HX is a chemical abrasive that may generate wound on cell surfaces. Alfalfa sprouts can be readily cultivated within a week at 20-27°C in humid environment. The objective of this study was to develop transgenic alfalfa sprouts as a novel type of plant transient expression system[4,5,6] with advantages of simple and rapid cultivation under efficient transformation condition.

Agroinfiltration[7] was carried out on alfalfa seeds at of 0, 1, and 2 days (D-0-S, D-1-S, and D-2-S, respectively) of germination as follows. Seeds were surface-sterilized by immersing in 0.4% sodium hypochlorite solution for 1 min, washed with sterile water, and soaked with sterile water for 24 h at 22°C. For GUS reporter gene transformation (D-0-S agroinfiltration), 100 μ*l* of 16 h-grown *Agrobacterium* cell cultures carrying pBI121 GUS expression vector were added to the imbibed seeds in 20 m*l* of sterilized distilled water, followed by vacuum-infiltration for 10 min. Transformed and non-transformed seeds were placed on a pre-wet paper towel at 27°C in the dark for growth. Agroinfiltration was identically performed to the germinating D-1-S and D-2-S. On day 5, growth of transgenic sprouts were stopped for histochemical

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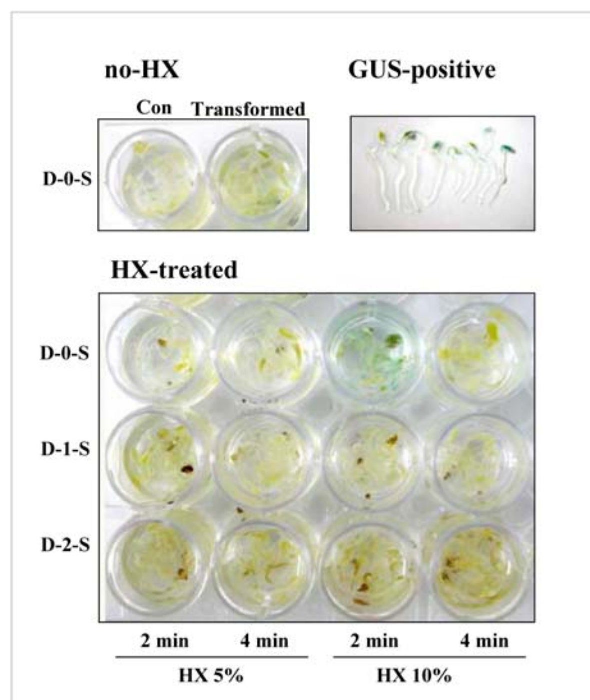
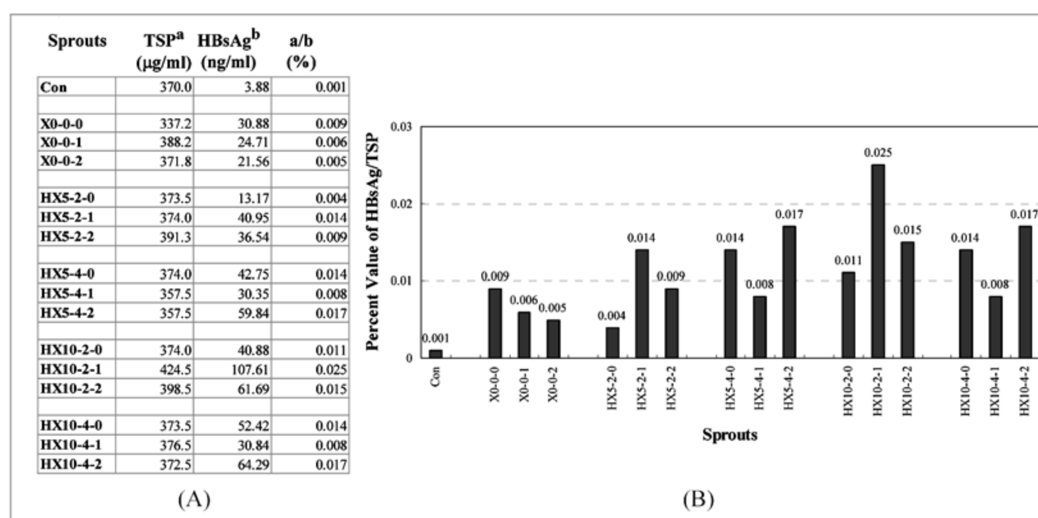


Fig. 1. Histochemical detection of GUS gene expression in transformed alfalfa sprouts. No-HX: Con (non-transformed sprouts) and transformed sprouts from pregerminated seeds (D-0-S). GUS-positive: closed view of GUS gene expressing sprouts. HX-treated: 5 or 10% hydrogen peroxide solution was treated for 2 or 4 min prior to agroinfiltration.

GUS gene analysis.[8] Sprouts were incubated with X-Glc solution at 27°C until blue color appeared and added with 70% ethanol to destain the chlorophyll pigment. Weak blue color was observed in transformed sprouts, whereas non-transformed (Con) sprouts showed no color change, implying the conventional agroinfiltration method was effective for alfalfa seeds transformation to some extent (Fig. 1). In the transformed sprouts, GUS-positive signal appeared mostly from hypocotyls and cotyledons but not from root. In a comparative experiment, HX was treated to D-0-S, D-1-S, and D-2-S for 2 or 4 min, followed by sufficient washing steps with sterilized water prior to agroinfiltration. GUS-positive signs appeared only in D-0-S sprouts treated with 10% HX for 2 min. The other HX-treated sprouts showed no or very faint GUS-positive sign. These results suggest that pre-germinated seeds are more disposed to transformation by agroinfiltratin and HX treatment could damage germinating seeds. GUS expression based on the histochemical detection, however, might lead to false-positive or -negative determination.[9]

Therefore, a different transformation experiment was performed to quantitate the transformation efficiency. Hepatitis B virus (HBV) is recognized as one of the world-threatening disease agents and recombinant HBV surface antigen (HBsAg) protein has been administered as vaccination worldwide.[10,11] A plant expression vector containing 0.7 kb



Sprouts	TSP[a] (μg/ml)	HBsAg[b] (ng/ml)	a/b (%)
Con	370.0	3.88	0.001
X0-0-0	337.2	30.88	0.009
X0-0-1	388.2	24.71	0.006
X0-0-2	371.8	21.56	0.005
HX5-2-0	373.5	13.17	0.004
HX5-2-1	374.0	40.95	0.014
HX5-2-2	391.3	36.54	0.009
HX5-4-0	374.0	42.75	0.014
HX5-4-1	357.5	30.35	0.008
HX5-4-2	357.5	59.84	0.017
HX10-2-0	374.0	40.88	0.011
HX10-2-1	424.5	107.61	0.025
HX10-2-2	398.5	61.69	0.015
HX10-4-0	373.5	52.42	0.014
HX10-4-1	376.5	30.84	0.008
HX10-4-2	372.5	64.29	0.017

Fig. 2. Evaluation of heterologous HBsAg protein was performed by ELISA for transformed alfalfa sprouts. A. Numbers attached to sprouts X (hydrogen peroxide not treated) and HX (hydrogen peroxide-treated) indicate, in the order of, hydrogen peroxide concentration (0, 5 or 10%), treatment duration (0, 2 or 4 min) and period of seed germination (0, 1, and 2). B. Values of a/b percent in (A) are converted into a bar graph.

DNA fragment which encoded HBsAg (pBIHBsAg) was constructed. PCR-amplified HBsAg DNA from pAM6 (ATCC 40101) was cloned into GUS DNA-deleted pBI121 (pBI121ΔGUS) (data not shown) and introduced into *Agrobacterium tumefaciens* LBA 4404 for alfalfa agroinfiltration. HX-treated and non-treated D-0-S, D-1-S, and D-2-S were subjected to agroinfiltration as described above. Sprouts were homogenized in the 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, and 1× protein inhibitor (Roche, Germany).[11] Clear homogenate collected following 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. Figure 2 A and B show total soluble protein (TSP) as determined by Bio-Rad Protein Assay system and percent value of HBsAg protein contained in TSP, respectively. In X0-0-0 (sprouts from D-0-S with no HX treatment), HBsAg protein content was 0.009% of TSP, whereas slight decreases were observed in X0-0-1 and X0-0-2 (sprouts from D-1-S and D-2-S, respectively), implying the pre-germinated seeds were slightly more efficient for the conventional agroinfiltration. On the other hand, Con showed 0.001% content probably as a result of the background outcome in our system. HX treatment resulted in different patterns of HBsAg expression from histochemical evaluation of GUS expression. The treatment enhanced the efficiency of *Agrobacterium* infection. HBsAg protein content of HX10-2-1 (sprouts of D-1-S with treatment of 10% HX for 2 mn) was more than twofold higher than that of X-0-0. Other sprouts, HX5-2-1, HX5-4-0, HX5-4-2, HX10-2-2, HX10-4-0 and HX10-4-2 also showed higher protein contents than X0-0-0. Although HX treatment appeared to be similarly effective in D-0-S, D-1-S, and D-2-S, more intense treatment on D-0-S (5%→10% and 2→4 min in this experiment) resulted in better influence on agroinfiltration. Overall, in sprouts of HX-treated D-1-S [HX(5, 10)-(2, 4)-1), HBsAg synthesis was compatible to or higher than that of X-0-0-0. In this case, 2 min HX treatment was more efficient than 4 min treatment under both 5 and 10% HX condition, suggesting that treatment duration was more critical in the range of 5-10% HX. In sprouts from D-2-S, increase in HBsAg expression could be obtained by more intense HX treatment. However, histochemical analysis gave different results; sprouts of HX10-2-0 with stronger GUS-positive sign showed marginally higher HBsAg content (0.011%) than X-0-0 (0.09%). Although further confirmation would be needed, we, at this point, can suggest that HX treatment-aided agroinfiltration to pre-germinated and germinating seeds improves alfalfa transformation effciency and, as a consequence, transient expression level.

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