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Note

Production of transgenic rice plants expressing *Dioscorea batatas* tuber lectin 1 to confer resistance against brown planthopper

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Abstract *Dioscorea batatas* tuber lectin 1 (DB1) is a storage protein isolated from yam tuber and has been shown to be a mannose-binding lectin. Here we produced transgenic rice plants expressing cDNA of *DB1* under the control of phloem-specific promoter of *rice sucrose synthase-1* gene. DB1 accumulated at a level of 0.07% of total soluble protein. We then evaluated its efficacy for brown planthopper (BPH). After releasing the first instar of BPH on the transgenic rice plants, the number of survived BPH adults was reduced up to 30% compared to that of wild-type rice. The number of the next generation BPH was suppressed to 22% on average in the seven most-resistant plants compared to that of wild-type rice plants when female adult BPH was inoculated. These results demonstrated that *DB1* is effective to confer BPH resistance in terms of decreased survival and fecundity.

Key words: *Dioscorea batatas* tuber lectin 1, insect resistance, planthopper, transgenic rice.

Three major sap-sucking pests of rice in Japan: brown planthopper (*Nilaparvata lugens*, BPH), whitebacked planthopper (*Sogatella furcifera*, WBPH) and small brown planthopper (*Laodelphax striatellus*) are known to cause severe damage to rice by sucking or acting as vectors for major viral disease. Recently, these pests have developed resistance to most commercial chemical pesticides (Matsumura et al. 2008). A transgenic approach has been explored to confer rice plants with resistance to such sap-sucking insects. One of the promising genes is encoding mannose-binding protein such as snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) and garlic leaf lectin (*Allium sativum* agglutinin from leaf; ASAL). Expression of *GNA* or *ASAL* in rice plants has been shown to confer substantial resistance to BPH, WBPH, and green rice planthopper in terms of increased insect mortality, retarded development and decreased fecundity (Rao et al. 1998; Nagadhara et al. 2003, 2004; Saha et al. 2006a; Yarashi et al. 2008). Mannose-binding proteins, in general, are known to bind to mannose-containing glycoproteins in the mid-guts of insects, and inhibit growth and development (Sauvion et al. 1996).

We have been focusing on *Dioscorea batatas* tuber lectin 1 (DB1), which has been isolated from yam tuber, *Dioscorea batatas* Decne., as a storage protein. DB1 is considered to be least non-harmful to human beings, as we Japanese eat raw tubers without boiling them. DB1 is a mannose-binding lectin (23 kDa) consisting of identical 12-kDa subunits. It has 58% and 48% amino-acid identity to GNA and ASAL, respectively, and is classified in the GNA-related lectin family (Gaidamashvili et al. 2004). The insecticidal properties of DB1 have been reported against moth larvae (*Helicoverpa armigera*). The rate of adults emerging from pupae was reduced to 33%, when fed on 0.01% (w/v) DB1 in an artificial diet (Ohizumi et al. 2009). We also demonstrated that $\geq$1 mg/ml DB1 in an artificial diet significantly decreased the survival and fecundity of green peach aphid, *Myzus persicaeca* (Kato et al. 2010). The number of survival aphids was reduced to 60% in transgenic tobacco expressing cDNA of *DB1* under the control of

Abbreviations: ASAL, *Allium sativum* agglutinin from leaf; BPH, brown planthopper; BS, bispyribac-sodium; DB1, *Dioscorea batatas* tuber lectin 1; GNA, *Galanthus nivalis* agglutinin; *mASL*, *mutant acetolactate synthase*; *RSs1*, *rice sucrose synthase-1*.

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Cauliflower mosaic virus 35S promoter or phloem-specific promoter of *rice sucrose synthase-1* gene. In this study, we produced transgenic rice plants expressing *DB1* under the control of *RSs1* promoter and evaluated their degree of resistance against BPH.

The *DB1* cDNA covering full-length ORF (accession no. AB513659) and the rice sucrose synthase-1 (*RSs1*) promoter (accession no. AJ401233; Shi et al. 1994) were obtained as described in Kato et al. (2010). The terminator of *RSs1* was PCR cloned using *RSs1*ter-1s primer (5′-TAA TGG AGG GGA AAA TAT GCA TCT TCA GCA GG-3′) and *RSs1*ter-2a-*Sac* primer (5′-GAGCTCATC TAC TAC AGC AGT AGA AAA GAT GCA ACC-3′; *Sac*I site is underlined) and connected to the 3′end of the *DB1* cDNA by blunt ligation. The resulting *RSs1* promoter::*DB1*::*RSs1* terminator cassette was taken as the *Sal*I–*Sac*I fragment and inserted by blunt-ligation to *Hin*dIII site of pSTARA binary vector (Fujioka et al. 2008), in which a mutant acetolactate synthase gene (*ALS*) was replaced by the G95A-mutated *ALS* (Okuzaki et al. 2007). This binary vector confers resistance to ALS-inhibiting herbicide, bispyribac-sodium (BS, Kumiai Chemical Industry Co., Ltd., Tokyo, Japan). The resulting construct was named *DB1*/*mALS* (Figure 1A) and was transferred into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993). Transformation of rice (*Oryza sativa* L. cv. Tachisugata) was carried out by the method of *Agrobacterium*-mediated transformation (Okuzaki et al. 2007). Tachisugata is a rice cultivar developed for the use of whole crop silage. The transformed calli were selected on a medium containing 0.25 μM BS and 40 mg/l Meropen (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan). Regenerated shoots were further selected on a rooting medium supplemented with 0.5 μM BS.

We obtained 23 plants (T_0 generation) showing resistance to 0.5 μM BS from calli induced from 2000 seeds. The presence of the introduced *DB1* cDNA was detected in 18 plants by PCR using the following primers; *DB1*-F1 primer (5′-CAG AAT GAC TGC AAC CTG GT-3′), and *DB1*-R2 primer (5′-ACC AAA GAT GGT GGC CTT AC-3′). T_1 plants derived from self-pollinated plants of no. 2, no. 3 and no. 6 were used for further analysis. The inheritance of the *DB1* was detected in all the T_1 plants investigated by PCR analysis (14 plants, 7 plants, 6 plants derived from line no. 2, no. 3 and no. 6, respectively), indicating the integration of multi-copies of DB1. For Southern blot analysis, genomic DNA was digested with *Bam*HI, which was cut once within the T-DNA, and probed with DIG-labeled *DB1* cDNA. Each plant showed 3 to 6 bands of different sizes, indicating integration of 3 to 6 copies of *DB1* (Figure 1B). The DB1 concentration in the total soluble protein in leaves was determined by comparing the intensity of bands which reacted with anti-DB1 polyclonal antiserum with those

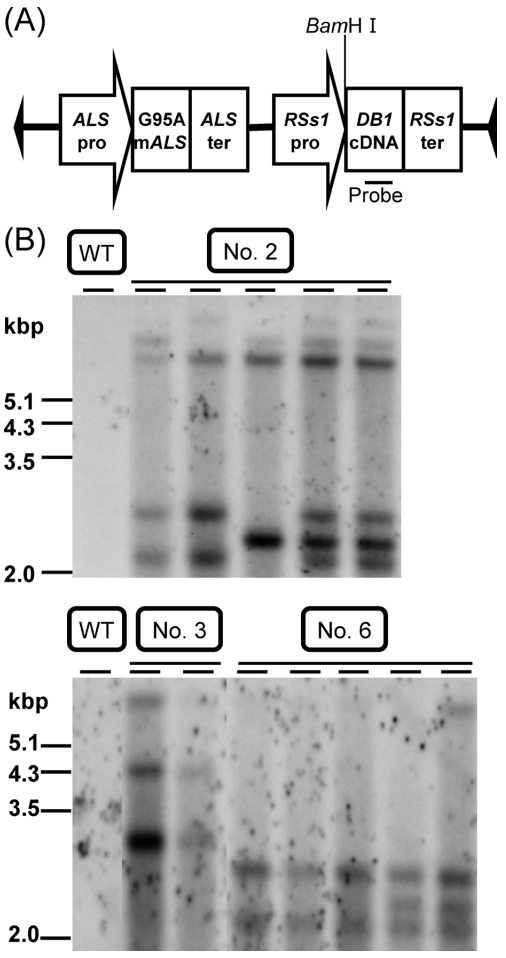

Figure 1. Transformation vector *DB1*/*mALS* (A) and Southern blot analysis to detect introduced *DB1*/*mALS* in T_1 plants (B). Each lane contains *Bam*HI-digested DNA isolated from a T_1 individual derived from self-pollinated transgenic lines no. 2, no. 3 and no. 6 or wild-type plants (WT). A probe is a part of *DB1* cDNA. *ALS*, *acetolactate synthase* of rice; *mALS*, G95A-mutated *ALS* gene; *RSs1*; *rice sucrose synthase 1* gene; *DB1*, *Dioscorea batatas tuber lectin 1*; pro, promoter; ter, terminator.

of series of known amounts of purified DB1, as described previously (Kato et al. 2010). An approximately 12-kd band corresponding to mature DB1 monomer was detected at the same position as that of the standard DB1 purified from yam tuber, indicating the proper processing of 16-kd DB1 premature protein in transgenic rice. DB1 accumulated at a level of 0.05 to 0.07% of total soluble protein in line nos. 2, 3 and 6 (Figure 2). The amounts of DB1 varied between each plant in the T_1 generation, probably because of the segregation of the integrated multi-copies of *DB1*.

Evaluation of BPH resistance in the T_1 generation was carried out on nine plants of line no. 2 and ten plants of line no. 6. The presence of *DB1* was confirmed in all the plants by PCR analysis. One-month old T_1 seedlings at 5th leaf stage were infested separately with 30 nymphs (1st instar) of BPH (accession Imari-07,

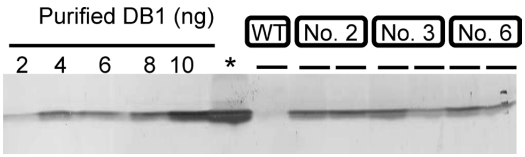

Figure 2. Western blot analysis of DB1 in leaves of each T_1 plant derived from self-pollinated transgenic lines no. 2, no. 3 and no. 6 (two plants per each transgenic line) or wild-type plants (WT). Twelve micrograms of total soluble protein were loaded for each lane. The DB1 concentration was determined by comparing the intensity of bands with those of known amounts of purified DB1 (2 to 10 ng). A star indicates a transgenic cultivar Taichung 65 with CaMV35S promoter::*DB1* (Kato et al. 2008).

which was collected at Imari city, Saga prefecture, Japan, as a pesticide, imidacloprid, resistant strain in 2007 and maintained in our laboratory). The average number of emerged adult BPH at 21st day after the inoculation was 16.8±2.2 (mean±SD) (n=9) on wild-type (WT) rice plants. In contrast, it was 12.3±5.1 on line no. 2 (n=9) and 13.9±3.9 on line no. 6 (n=10) (Figure 3). High variance of the BPH survival rates in each transgenic line would be attributed to uneven accumulation of DB1 due to the segregation of multiple copies of the transgene in the T_1 generation. Although no significant difference of the survival rates was observed among line no. 2, no. 6 and WT (ANOVA after arcsine transformation, F=2.758, df=2, p=0.083), some individuals of these transgenic lines obviously showed the lower survival of BPH nymphs. The most prominent plant showed a reduced number of BPH up to 30% of the WT average. We considered that DB1 had an entomotoxic effect on BPH mortality when expressed in rice. To investigate the effect of DB1 on fecundity, 20 female adults were newly inoculated on the same plants after all the BPH used for the above experiment was washed away. The numbers of the next generation nymphs (1st to 5th instar) and adults were counted on the 14th and 27th day. On the 27th day, all of the transgenic plants except one showed lower numbers of BPH than WT, although statistically significant difference was not obtained among line no. 2, no. 6 and WT (ANOVA after $\log_e(x+0.5)$ transformation, F=2.433, df=2, p=0.130). The average number of BPH was 247.0±15.6 (mean±SD) (n=4) on wild-type (WT) rice plants. In contrast, it was 122.9±85.5 on line no. 2 (n=7), and 77.8±53.6 on line no. 6 (n=4) (Figure 4). The relatively lower number of BPH was recorded on some individuals of the transgenic lines: 6 to 99 BPH on four plants of line no. 2, and 32 to 68 BPH on three plants of line no. 6 (Figure 4). The number of BPH was reduced to 2.4 to 37% with an average of 22%, compared to the WT average, in these seven transgenic plants, which might carry an effective copy of the transgene for higher accumulation of DB1. We considered the DB1 was effective in reducing fecundity of BPH.

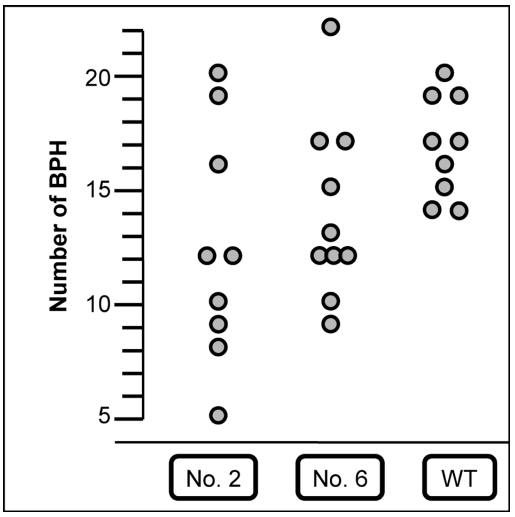

Figure 3. The number of BPH adults who survived on each transgenic T_1 plant on the 21st day after inoculating 30 nymphs (1st instar) of BPH. Each circle indicates an individual T_1 plant derived from self-pollinated transgenic lines no. 2 and no. 6 or wild-type plants (WT).

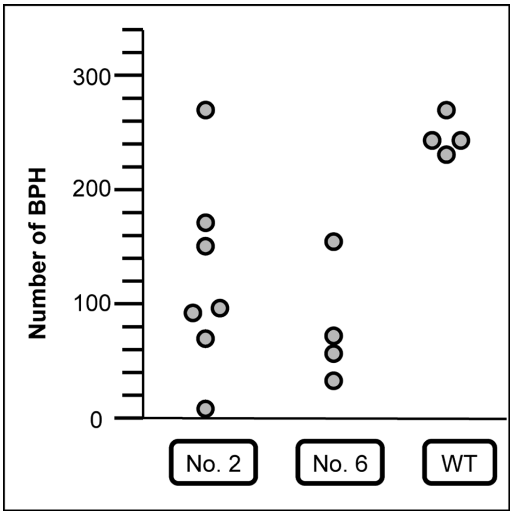

Figure 4. The number of the next generation nymphs and adults of BPH on each transgenic T_1 plant on the 27th day after inoculating 20 female adults of BPH. Each circle indicates an individual T_1 plant derived from self-pollinated transgenic lines no. 2 and no. 6 or wild-type plants (WT).

Our current study demonstrated the efficacy of *DB1* to confer BPH resistance in rice in the same manner as reported for GNA and ASAL. In the study of transgenic rice with *RSs1* promoter::*ASAL*, the survival rate of BPH was reported to be reduced to 40 to 46%, and the fecundity was reduced to 28 to 37% (Saha et al. 2006a). *ASAL* has been also reported to confer the green leafhopper-vectored rice tungro virus (Saha et al. 2006b). Acquisition of resistance against the planthopper-vectored virus can be expected in the transgenic rice plants expressing DB1. *RSs1* promoter has the advantage of maximizing expression of the

insecticidal protein at the site of attack by sap-sucking insects, while minimizing it elsewhere in plants (Shi et al. 1994; Nagadhara et al. 2003, 2004; Saha et al. 2006a, b). The T-DNA region of our current study is composed of all rice genes except for *DB1* from *Dioscorea batatas*. The selection marker is the G95A-mutated *ALS* gene from rice, which confers ALS-inhibiting herbicide, BS. Our transformation vector will be useful for producing stacked GM crops resistant to both herbicide and the sap-sucking pests with a merit of being least non-harmful to human beings. The rice cultivar, Tachisugata, used in this study is one developed for the use of whole crop silage for livestock. The transgenic Tachisugata produced in this study can be used as a practical foliage crop after selecting the stable transgenic lines homozygous for the introduced *DB1*/*mALS*.

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