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Original Paper

Expression of gene for *Dioscorea batatas* tuber lectin 1 in transgenic tobacco confers resistance to green-peach aphid

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Abstract *Dioscorea batatas* tuber lectin 1 (DB1) is a storage protein isolated from yam tuber, and is shown to be a mannose-binding lectin. It has 58% amino-acid identity to insecticidal snowdrop bulb lectin GNA. In this study, we demonstrated that $\geq$1 mg ml^{-1} DB1 in an artificial diet significantly decreased the survival and fecundity of green peach aphid, *Myzus persicaeca*. We produced transgenic tobacco plants expressing cDNA of DB1 under the control of Cauliflower mosaic virus 35S promoter (35S-DB1) or phloem-specific promoter of rice sucrose synthase-1 gene (RSs1-DB1), and evaluate the degree of aphid resistance in whole plant bioassays. The number of survival aphids was reduced to 60% in transgenic lines with 35S-DB1 and RSs1-DB1, which accumulated DB1 at a level of 1.8% and 0.25%, respectively, of total soluble protein. Our results indicate that DB1 can be used to enhance resistance to sap-sucking insects in transgenic crops.

Key words: Aphid, *Dioscorea batatas* tuber lectin 1, insect resistance, transgenic tobacco.

Genetically modified (GM) crops are now cultivated worldwide, and insect-resistant crops accounted for 18% of GM-cultivated area in 2007 according to a report of the International Service for the Acquisition of Agri-Biotech Applications (ISAAA Brief 37-2007; http://www.isaaa.org/resources/Publications/briefs/37/). All insect-resistant GM crops contain a *Cry* gene encoding Bt toxin of *Bacillus thuringiensis*. The *Cry* gene has been shown to be useful for controlling various chewing insects such as lepidopteran and coleopteran larvae. Bt transgenic crops, however, are known to be susceptible to sap-sucking insects such as aphids and planthoppers (Rao et al. 1998). In contrast, snowdrop bulb lectin (*Galanthus nivalis* agglutinin; GNA), which was isolated from an ornamental monocotyledonous plant, has been successfully used to enhance resistance to sap-sucking insects in transgenic tobacco (Hilder et al. 1995), maize (Wang et al. 2005) and rice (Nagadhara et al. 2003; 2004; Rao et al. 1998). GNA belongs to the mannose-binding lectin family. Mannose-binding proteins, in general, are known to bind to mannose-containing glycoproteins in the mid-guts of insects, and to inhibit growth and development (Sauvion et al. 1996).

DB1 has been isolated from yam tuber, *Dioscorea batatas* Decne., as a storage protein. DB1 is a monnose-binding lectin (23 kDa) consisting of identical 12-kDa subunits. It has 58% amino-acid identity to snowdrop lectin GNA and is, thus, 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 has been reduced to 33%, when fed on 0.1 mg ml^{-1} DB1 in an artificial diet (Ohizumi et al. 2009). A gene for DB1 is, therefore, expected to be useful for the production of insect-resistant crops.

In this study, we first examined the insecticidal activity of DB1 protein against aphids using an artificial diet assay. Then we produced transgenic tobacco expressing DB1 cDNA under the control of Cauliflower mosaic virus (CaMV) 35S promoter or rice sucrose synthase-1 (RSs1) promoter. The RSs1 promoter has been reported to be a phloem-specific promoter and is thought to be more effective on sap-sucking insects and minimize any potential undesirable accumulation of the protein in other parts of plants (Rao et al. 1998). The insecticidal activity of DB1 against aphids was demonstrated in the whole plant assay of the transgenic plants.

This article can be found at http://www.jspcmb.jp/

Materials and methods

Artificial diet bioassays

Green peach aphid (*Myzus persicae*) was obtained form JT (Japan Tobacco Inc. Japan). Aphids were maintained on mature plants of *Nicotiana tabacum* cv. Petit Havana SR1 in a controlled environment growth chamber, 25 ± 1ºC, 16L/8D.

The artificial diet bioassays for aphids was carried out as described by Powell et al. (1993) using an artificial diet for aphids (Dadd and Mitter 1966). DB1 was incorporated at 2, 1 and 0.1 mg ml^{-1} in the artificial diet. Controls were set as treatment with feed using an artificial diet containing 0 mg ml^{-1} DB1 or 1 mg ml^{-1} BSA, and treatment with no diet (no diet controls). Ten adult apterous aphids were transferred from the host plant to glass petri-dishes. Each petri-dish was sealed with a stretched Parafilm (Pechiney Plastic Packaging Inc., USA), an 500 μl artificial diet was placed on top of the stretched Parafilm, and then the artificial diet was covered with another stretched Parafilm. The diet and Parafilm were changed daily. The bioassays were conducted in an environmental growth chamber (25 ± 1°C, 16L/8D), and the number of surviving adults and newborn nymphs was counted with four sets of replication. A statistical analysis was carried out using the Excel Statistics software.

Transformation of tobacco

The nucleotide sequence for a DB1 isoform, DB1 (Leu86), under the accession no. AB178475 in DDBJ was revealed to be lacking a part of 5′ signal sequence. PCR for 5′ RACE was carried out to obtain full length cDNA using yam tuber mRNA (Ohizumi et al. 2009) and the Marathon cDNA Amplification Kit (BD Bioscience Clontech). The nucleotide sequence of full length open reading frame (ORF) of DB1 cDNA was deposited to DDBJ (accession no. AB513659). It encodes 172 amino-acid protein with a predicted targeting-signal sequence to endoplasmic reticulum.

The cDNA covering full-length ORF was PCR cloned into pGEM T-vectors (Promega, Madison, USA) using KOD+ polymerase (Toyobo, Osaka, Japan) and primers XB/NDB1 F 5′-TCTAGAGGATCCATGGCTAACCCAGGAGCA-3′ (*Xba* I and *Bam* HI sites are underlined) and S/DB1 R 5′-GAGCTC-TCACTTGTTGACGACC-3′ (*Sac* I site is underlined). The PCR products were digested with *Bam* HI/*Sac* I and inserted into *Bam* HI/*Sac* I sites of pBE2113 (Mitsuhara et al. 1996), which contained CaMV 35S promoter with doubled enhancer and omega sequence of tobacco mosaic virus for enhanced expression. The resulting construct, 35S-DB1, was used for strong and constitutive expression of the DB1 gene. For phloem-specific expression of DB1, the rice sucrose synthase-1 (RSs1) promoter (accession no. AJ401233) was employed as described (Shi et al. 1994). The promoter sequence (3 kb) was amplified from rice cv. Taichung 65 using KOD+ polymerase and specific primers Sal/RSs1 F 5′- GTCGACCTTTCGTGAC-TTGTTTTCGC-3′ (Sal I site is underlined) and Bam/RSs1_R 5′-GGATCCTAGCTTGGCAGCCAT-3′ (*Bam* HI site is underlined), and was subcloned into pGEM-T vector. Then RSs1 promoter was inserted into *Sal* I/*Bam* H I sites of pBI101H, which contained the hygromycin resistance cassette (Ariizumi et al. 2002). The GUS gene in pBI101H was replaced by DB1 cDNA at *Bam* HI/*Sac* I sites. The resulting construct was named RSs1-DB1. These constructs were transferred into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993). Transformation of tobacco (*Nicotiana tabacam* L. Petit Havana SR1) was carried out using a standard leaf-disk method. The transformants were selected on shoot-inducing medium containing 50 mg l^{-1} kanamycin and 1 mg l^{-1} meropen (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan).

Western blot analysis

The total soluble protein (TSP) was extracted from leaves of T_0 and T_1 transgenic tobacco in 50 mM Tris-HCl (pH 7.0). The concentration of extracted protein was determined using the Bio-Rad protein assay kit. Approximately ~10 μg of TSP was separated on 12% SDS-PAGE and transferred onto PVDF membrane (0.45 μm, Millipore, USA). The membrane was reacted with anti-DB1 polyclonal antiserum at 1 : 10,000 dilution (Ohizumi et al. 2009) and anti-rabbit IgG Alkaline phosphatase conjugate (Promega, USA) as secondary antibody at 1 : 5,000 dilution. Bound secondary antibody was detected using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Wako Chemicals, Tokyo, Japan). The DB1 concentration in TSP was determined by comparing the intensity of bands with those of series of known amounts of purified DB1.

Whole plant bioassays

Transgenic tobacco plants (T_1) with accumulated DB1 and untransformed wild-type tobacco (WT) were grown in 5-inch pots in a controlled environmental growth room (25 ± 1ºC, 16L/8D). When plants were about 20 cm tall, each plant was transferred into a 30×30×40 cm plastic case and infested with 5 adult apteraous aphids. The numbers of aphids on each plant were counted daily. Four plants were tested for each transgenic line.

Results

Effect of DB1 on green peach aphid in artificial diet bioassays

The insecticidal activity of DB1 against green peach aphid was tested at 2, 1 and 0.1 mg ml^{-1} DB1 in the artificial diet. The number of survivors was counted at one to seven days of feeding (Figure 1). The number of survivors was greatly reduced to almost zero after 3 days in the no diet control, while it was slightly reduced when administrated with 1 mg ml^{-1} BSA and 0 mg ml^{-1} DB1. Addition of 1 mg ml^{-1} and 2 mg ml^{-1} DB1 significantly ($P<0.01$) reduced the number of survivors in comparison with treatments containing 1 mg ml^{-1} BSA and 0 mg ml^{-1} DB1. No aphids survived after 7 days of feeding with 2 mg ml^{-1} DB1.

Corrected mortality was calculated at the 3rd day when the number of survivors in the no diet control reached zero as follows: (number of survivors in 0%

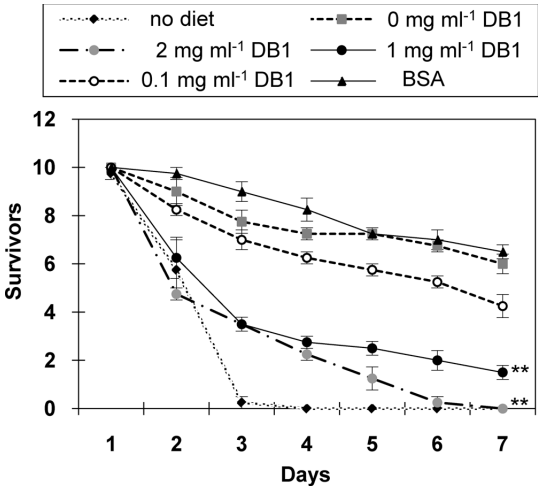

Figure 1. The number of survivors of aphids in artificial diet bioassay (n=4). DB1 was incorporated at 2, 1 and 0.1 mg ml^{-1} in the artificial diet. Controls were set as treatment with feed using an artificial diet containing 0 mg ml^{-1} DB1 or 1 mg ml^{-1} BSA, and treatment with no diet. The survival rate was statistically analyzed using the Kaplan-Meier method and significance of differences was analyzed by the log-rank test. ** implies a significant difference between control (0 mg ml^{-1} DB1) and each treatment at $P<0.01$.

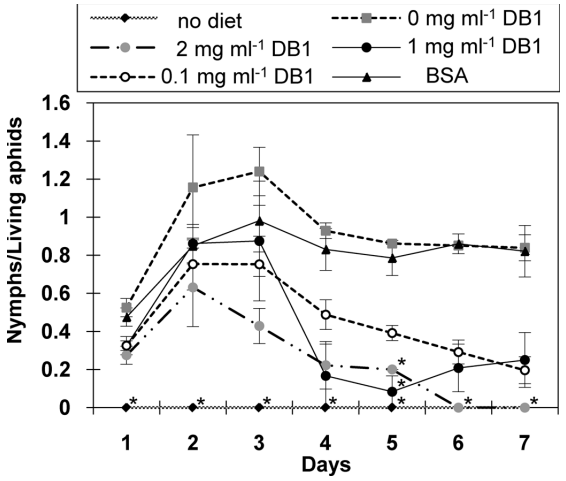

Figure 2. The number of nymphs per number of living aphids in artificial diet bioassay (n=5). DB1 was incorporated at 2, 1 and 0.1 mg ml^{-1} in the artificial diet. Controls were set as treatment with feed using an artificial diet containing 0 mg ml^{-1} DB1 or 1 mg ml^{-1} BSA, and treatment with no diet. * implies a significant difference between control (0 mg ml^{-1} DB1) and each treatment at $P<0.05$ by Steel's test after Kruskal-Wallis test.

DB1 condition—number of survivors in the condition of designated concentration of DB1)/(Number of survivors in 0% DB1 condition)×100 (Abbot1925). Corrected mortality was 70% in 2 mg ml^{-1} DB1 condition, 62% in 1 mg ml^{-1} DB1 and 13% in 0.1 mg ml^{-1} DB1. These results indicated that DB1 has insecticidal activity in a concentration-dependent manner.

The number of nymphs was also counted every day. The number of nymphs per number of living aphids was around 0.92 and 0.83 when fed with 0 mg ml^{-1} DB1 diet and 1 mg ml^{-1} BSA-containing diet for 4 days respectively, whereas it was reduced to 0.48 in the case of 0.1 mg ml^{-1} DB1 condition, and approximately 0.2 in cases of 1 mg ml^{-1} and 2 mg ml^{-1} DB1 (Figure 2). The difference was significant ($P<0.05$) at the 5th day. These results indicate that DB1 decreased the survival and fecundity of green peach aphid.

Transgenic tobacco accumulating DB1

We produced several lines of transgenic tobacco plants with 35S-DB1 or RSs1-DB1. The accumulated DB1 in leaves was detected using western blot analysis. An approximately 12-kd band corresponding to mature DB1 monomer was detected at the same position as that of standard DB1 purified form yam tuber, indicating the proper processing of 16-kd DB1 premature protein in transgenic tobacco (Figure 3). In the case of transgenic lines with 35S-DB1, DB1 accumulated at a level of 2.4% of total soluble protein in plant no. 35S-1, 2.0% in no. 35S-6 and 1.4% in no. 35S-7. The progeny of no. 35S-1 (nine plants of T_1 generation with 35S-DB1) also contained DB1 at almost the same level with an average

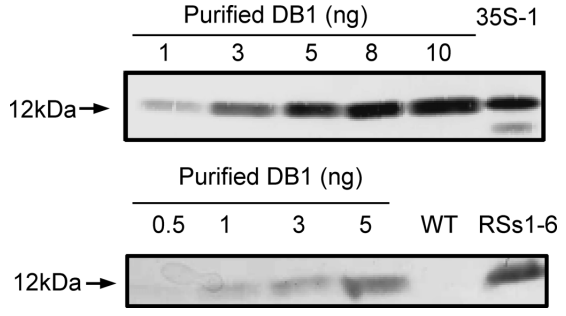

Figure 3. Western blot analysis of DB1 in leaves of transgenic tobacco with 35S-DB1 (35S-1) and RSs1-DB1 (RSs1-6) and non-transgenic tobacco (WT). The DB1 concentration in transgenic tobacco was determined by comparing the intensity of bands with those of series of known amounts of purified DB1 (0.5 to 10 ng). Five μg of total soluble protein were loaded for each transgenic tobacco and WT.

of 1.8%.

In the case of transgenic lines with RSs1-DB1, DB1 accumulated at a level of 0.1% of total soluble protein in plant nos. RSs1-2 and RSs1-3, and 0.25% in no. RSs1-6. The progeny of no. RSs1-6 (nine plants of T_1 generation with RSs1-DB1) also contained DB1 at the same level of 0.25% on average.

Effect of DB1 on green peach aphid in whole plant bioassays

Four T_1 plants of the each transgenic line, no. 35S-1 and no. RSs1-6, in which accumulation of DB1 was confirmed, were used for evaluation of the degree of aphid resistance in whole plant bioassays. Transgenic line no. 35S-13 accumulating no detectable DB1 and non-transformed WT were used as a negative control. The number of aphids per plant was counted up to 14

days of bioassays (Figure 4). At the 14th day, the number of aphids on average was 481 ± 21 in plant no. 35S-1, and 492 ± 14 in plant no. RSs1-6, while it was 786 ± 72 in WT and 755 ± 18 in 35S-13 (negative control). The number of aphids per plant was significantly ($P<0.05$) reduced to 60% in DB1-accumulating tobacco compared to non-accumulating tobacco. No differences were found in the number of aphids between the 35S-DB1 line and the RSs1-DB1 line. This result clearly demonstrated the transgenic tobacco expressing DB1 enhanced resistance to green peach aphid.

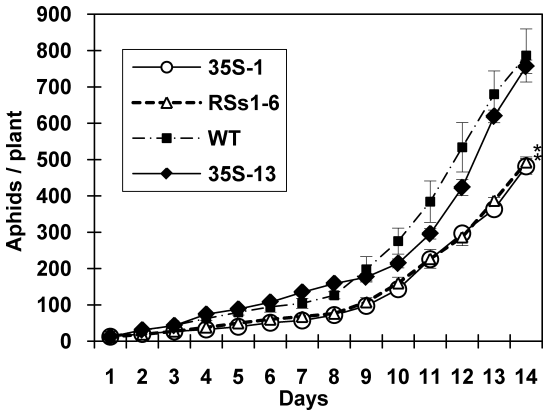

Figure 4. The number of aphids per transgenic tobacco plant with 35S-DB1 (35S-1), RSs1-DB1 (RSs1-6) and WT ($n=4$). DB1 accumulated at a level of 1.8% of total soluble protein in the 35S-1 line and 0.25% in the RSs1-6 line. No. 35S-13 is a negative control accumulating no detectable DB1. * implies a significant difference between WT and each transgenic plant at $P<0.05$ by Steel's test after Kruskal-Wallis test.

Discussion

An LD50 for DB1 in artificial diet was estimated to be 0.67 mg ml^{-1} at the 4th day and 0.27 mg ml^{-1} at the 7th day based on Probit analysis. Concentrations more than 1 mg ml^{-1} caused significant reduction of mortality and fecundity. The corrected mortality of 1 mg ml^{-1} DB1 was 62%. The toxicity of lectin against green peach aphid has also been reported for snowdrop lectin GNA (Hilder et al. 1995). The reported corrected mortality of 1 mg ml^{-1} GNA was 33%. Thus the toxicity of DB1 was similar to that of GNA. GNA has been reported to show insecticidal activity against other sap-sucking insects. The corrected mortality of 1 mg ml^{-1} GNA against brown planthopper (*Nilaparvta lugens*) and green rice planthopper (*Nephotterix cinciteps*) was reported to be 79% and 87%, respectively (Powell et al. 1993). DB1 is expected to have entomotoxic effects against such planthoppers.

We employed CaMV35S promoter and RSs1 promoter to direct DB1 expression in tobacco. Transgenic tobacco accumulating GNA up to 2.5% total soluble protein, which was expressed under the control of CaMV35S promoter, has been shown to decrease the number of living green peach aphids to 56% in leaf disk bioassays (Hilder et al. 1995). The insecticidal effect of DB1 on aphid in transgenic tobacco is similar to that of the previous report of GNA. Similar results have been also reported for garlic lectin (*Allium sativum* agglutinin from leaf; ASAL), which shares a 48% amino- acid identity with DB1. Expression of ASAL under the control of the phloem-specific promoter *Asus1* from *Arabidopsis thaliana* has been reported to protect tobacco plants against the tobacco aphid (*Myzus nicotianae*) with a reduction of 40% of reproduction capacity (Sadeghi et al. 2007). GNA and ASAL have been shown to confer substantial resistance to brown planthopper, green rice planthopper, and whitebacked planthopper, in terms of increased insect mortality, retarded development and decreasing fecundity, when expressed in transgenic rice (Nagadhara et al. 2003; 2004; Saha et al. 2006; Yarasi et al. 2008). Recently, virus resistance has been also reported in transgenic rice expressing ASAL under the control of RSs1 promoter (Saha et al. 2006). We are now investigating the resistance against planthoppers and rice tungro virus in transgenic rice plants expressing DB1.

RSs1 promoter has also been shown to direct phloem-specific expression of beta-glucuronidase and GNA in transgenic tobacco (Shi et al. 1994). RSs1 promoter has also been used to drive GNA in transgenic rice (Nagadhara et al. 2003; 2004) and maize (Wang et al. 2005), and to drive garlic lectin, ASAL, in transgenic rice (Saha et al. 2006). 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. Our current study demonstrated that the level of aphid resistance is the same between the RSs1-DB1 and 35S-DB1 lines, although the amount of DB1 per soluble protein extracted from a whole leaf of the RSs1-DB1 line was approximately one-seventh of that in the 35S-DB1 line, suggesting that the amount of DB1 in phloem might be almost identical. Our results confirmed the effectiveness of RSs1 promoter.

DB1 was isolated from yam tubers of Japanese cultivar, which are generally eaten as raw tubers without boiling. DB1, therefore, is considered to be least non-harmful to human beings. Taking our current study into consideration, the DB1 gene would be useful for producing safe GM crops with resistance to sap-sucking insects.

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