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Transgenic Rice Expressing Snowdrop Lectin (*Galanthus nivalis* agglutinin; GNA) Shows Resistance to Rice Brown Planthopper (*Nilaparvata lugens* Stål)

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Transgenic rice plants with increased resistance to rice brown planthopper (*Nilaparvata lugens* Stål) were generated by particle bombardment-mediated transformation of plants with a gene encoding snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) under control of the rice Rubisco small subunit (*rbcS*) promoter.. A large number of transgenic rice plants containing the *GNA* gene were generated. The integration, expression, and inheritance of this gene in the R_1 and R_2 generations were demonstrated by Southern and western blot analyses. The plants contained one to five copies of the transgene. The GNA protein comprised approximately 0.01-2.0% of total soluble protein in the R_1 and R_2 transgenic plants. Insect bioassays and feeding studies showed that the GNA protein expressed in the R_2 transgenic rice plants reduced the survival of brown planthoppers. The introduction of *GNA* into rice plants therefore can help to control insect pests.

Key words : Brown planthopper, *Galanthus nivalis* agglutinin (GNA), insect resistance, particle bombardment, transgenic rice plants

Introduction

Rice, one of the most important food crops worldwide, grows in diverse soils and climates and is best adapted to warm, humid environments in which insects proliferate. Rice grain yields are often reduced due to direct damage by insects. Additionally, several devastating viral diseases, including rice tungro and yellow dwarf disease, are transmitted by insects [17]. Brown planthopper (BPH, *Nilaparvata lugens* Stål) is a serious rice insect pest that causes severe physiological damage by feeding on the plant. BPH is also a vector for viruses such as grassy stunt virus and ragged stunt virus [17]. BPH reduces rice yields by 53.8 kg per hectare in Southeast Asia, an estimated annual loss of 248.5 million USD [10]. Conventional methods used to control insects that feed on rice primarily involve the use of chemical insecticides, which are currently under public scrutiny due to food safety and environmental concerns. Thus, plant breeders have sought new techniques to minimize yield loss, including introgression and molecular tagging of genes from *Oryza australiensis* into cultivated rice (*O. sativa*), to increase resistance to BPH [12]. In addition, extensive research has focused on identifying potential entomotoxic proteins, which could be genetically engineered into rice plants to control pests, thereby replacing chemical pesticides [21]. Indeed, genetically modified crops have been produced that express entomotoxic proteins of either bacterial or plant origin [7]. Two major groups of plant-derived genes, inhibitors of digestive enzymes (protease and amylase) and lectins, have been used to confer resistance to homopteran insect pests [26]. Recently, bioassay studies of protease/amylase inhibitor-expressing plants show that these genes can affect plant-eating insects [8,15]. Other studies have used bioassays based on an artificial diet feeding system [21] to show that carbohydrate-binding plant lectins are highly antinutritional and/or toxic to various phloem-feeding insect pests. A mannose-specific lectin derived from the snowdrop (*Galanthus nivalis* agglutinin; GNA) is toxic to a number of important insect pests, including homopterans, coleopterans, and lepidopterans, when incorporated into artificial diets. This tetrameric protein, comprising identical subunits of approximately 12 kDa, specifically binds to α-1,3- or 1,6-linked D-mannose residues on carbohydrates [32]. Although GNA is toxic to insects, it is non-toxic to mammals [22]. Because of the potent entomotoxic properties exhibited by GNA, the *GNA* gene has been introduced into a number of plants, including tobacco [11], potato [1,6], tomato [33], wheat [18,28]

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and maize [34]. *GNA* has also been introduced into rice [2,5,20,23,27,29,30], and transgenic rice plants containing this gene show increased resistance to BPH in bioassays and feeding tests [5,23,29]. This gene has negligible effects on the development, survival, and fecundity of beneficial insects [4]. Here, we report the generation of transgenic rice plants expressing *GNA* using the method of particle bombardment. The protein was expressed in a tissue-specific manner, and the transgenic plants were resistant to BPH. The production of transgenic plants using insecticidal genes such as *GNA*, combined with integrated pest management, is a promising technique for the control of damaging rice pests.

Materials and Methods

Plasmid

The gene construct containing the *GNA* coding sequence from pLECGNA2 was previously described [31]. To achieve tissue-specific expression of *GNA* in the photosynthetic tissue of rice plants, the *GNA* coding sequence was fused to the rice Rubisco small subunit (*rbcS*) promoter and *nos* terminator in pBluescript-SK. The resulting plasmid was designated pIP840 (Fig. 1). The pDM302 vector contains the phosphinothricin acetyltransferase (*bar*) gene to enable selection on medium containing phosphinothricin (Fig. 1). Both constructs were used for co-transformation of rice.

Rice transformation

Transformation of rice microcalli using particle bombardment, and the recovery of transgenic rice plants, was performed as described by Lee et al. [16]. Callus was initiated from mature seeds of the japonica cultivar, Nagdongbyeo. The microcalli were subjected to microprojectile bombardment using a Biolistic Particle Delivery PDS-1000/He system (Bio-Rad). The bombarded microcalli and transformants were selected on media containing phosphinothricin (15 μg ml-1) [14]. Actively proliferating calli were transferred to regeneration medium for 4-8 weeks, followed by transfer to 1/2MS medium [19]. Regenerated plants were grown to maturity in a greenhouse.

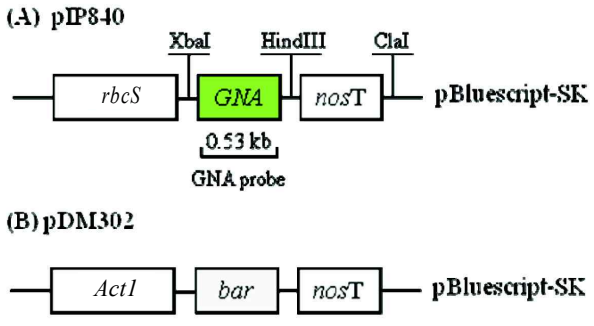

Fig. 1. Plasmids used to express the *GNA* gene in transgenic rice plants. The *GNA* gene in pIP840, which contains a *nos* 3′ region, is regulared by the *rbcS* promoter. In pDM302, the bacterial phosphinothricin acetyltransferase gene (*bar*), which is regulated by the *Act1* promoter, served as a selectable marker for rice transformation.

Southern blot analysis

Genomic DNA was extracted from leaf and stem tissues of transformed rice plants based on Dellaporta *et al.* [3] with modofication. Five μg of genomic DNA from each sample was digested with either *Hind* III/*Xba* I or *Cla* I (Fig. 1), fractionated on a 0.8% agarose gel, denatured, and transferred onto a nylon membrane. The membrane was pre-hybridized for 1 hr at 65°C in a solution containing 0.25 M sodium phosphate (pH 7.4), 1 mM EDTA, 1% BSA and 7% SDS, and hybridized in the same solution with ^{32}P-labeled *GNA* probe. Then the membrane was washed twice with 2x SSC solution at room temperature and once with 0.1 x SSC solution containing 0.1% SDS at 60°C for 15 min each time [24].

Western blot analysis

Protein was extracted from leaves of R_1 and R_2 transgenic rice plants. The soluble fraction (20 μg) was separated on duplicate 12% SDS-polyacrylamide gels [13]. One gel was stained with Coomassie blue, while the proteins on the other gel were transferred to a nitrocellulose membrane by electroblotting. The nitrocellulose membrane was treated with blocking solution [Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 and 6% (w/v) nonfat dry milk] in order to reduce non-specific binding. The membrane was incubated for 1 hr with a 500- fold dilution of anti-GNA antibody. After washing with TBS solution containing 0.1% (v/v) Tween 20, the membrane was incubated with a 1/1000 dilution of the peroxidase-labeled secondary antibody (Sigma) for 1 hr. Membrane was washed again in TBS and developed with enhanced chemiluminescence (ECL) Western blotting kit (Amersham).

Insect bioassay

The insecticidal activity of the transgenic plants toward BPH was assayed using a feeding chamber as previously described [9]. Two-month-old R_2 plants, homozygous for the

GNA gene, which strongly expressed GNA (as determined by western blot analysis) were used in this assay. R_2 plants derived from a non-transgenic R_1 plant, which had lost the *GNA* gene due to Mendelian segregation, were used as a negative control. The plants were transferred to a feeding chamber into which 12 second-instar nymphs were released. The insects fed continously for 8 days at 24°C under a 14/10 hr light/dark regime. The number of surviving insects was recorded daily. To determine the effects of BPH on transgenic and control plants, control plants and high GNA-expressing R_2 plants were transferred to wood-net chambers (60×45×30 cm), and 100 second- and third-instar nymphs were released onto the plants. The amount of damage to each plant was determined 4 weeks after infestation.

Results and Discussion

Production of fertile transgenic rice plants

Transgenic rice plants were produced by co-transformation with plasmids pDM302 (containing the *bar* gene, which encodes a selectable marker) and pIP840 (containing the *rbcS* promoter fused to the *GNA* coding sequence) by particle bombardment. These plasmids were introduced into embryogenic calli of the japonica rice variety 'Nagdongbyeo'. Several independent transformation experiments were performed, both with and without plasmid (control). Calli bombarded with recombinant plasmids were selected for 4 weeks on culture medium containing 15 μg/ml phosphinothricin. A total of 53 independent transgenic plants were generated. Most of these plants were derived from independent calli. Forty-six plants were fertile, whereas the remaining plants were sterile.

Integration of *GNA* gene in transgenic rice plants

The presence of the *GNA* gene in the transgenic plants was confirmed by Southern blot analysis (Fig. 2). Total genomic DNA was isolated from the leaves of seven randomly-chosen phosphinothricin-resistant plants, and one untransformed plant. The genomic DNA was then digested with *Cla* I (A lanes) or *Hind* III/*Xba* I (B lanes) and probed with a 0.53-kb *Hind* III/*Xba* I fragment containing the entire *GNA* expression cassette (*rbcS* promoter, *GNA* gene and *nos* terminator). *Cla* I cleaves the intact plasmid, pIP840, at a single site to generate a 6.0-kb linear molecule. The size of the *GNA*-hybridizing fragment should indicate the distance from the *Cla* I site of the integrated plasmid to the nearest

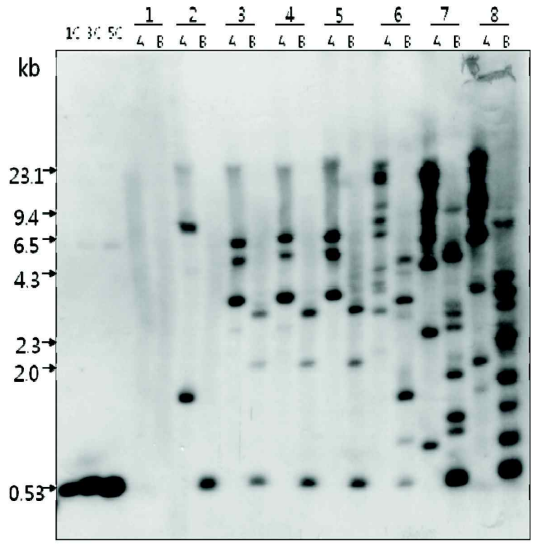

Fig. 2. Southern blot analysis of genomic DNA from transgenic plants. DNA (5 μg) from transgenic lines containing pIP840 was digested with *Cla* I (A) or *Hind* III/*Xba* I (B), resolved on a 0.8% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled *GNA* probe. 1, DNA from wild-type Nagdongbyeo plant; 2-8, transgenic plants. 1C, 3C and 5C contain the *GNA* coding sequence in amounts equivalent to 1, 3 or 5 copies per haploid rice genome.

Cla I site in the rice genome. *Hind* III/*Xba* I digestion should produce intact *GNA* fragments. All seven phosphinothricin-resistant plants showed a 0.53-kb hybridization signal corresponding to the size of the intact *GNA* coding sequence (Fig. 2, lanes 2–8, B), while no signal was detected in DNA isolated from the untransformed plant (lane 1). The phosphinothricin-resistant phenotype, therefore, corresponded to the presence of the intact *GNA* gene. In addition to the 0.53-kb fragment, two additional bands in lanes 3B, 4B and 5B, which suggest that the DNA in these lanes may have come from plants that were regenerated from the same transformed callus. After hybridization with the 0.53-kb *GNA* probe, DNA from the untransformed control plant did not produce any detectable signal (Fig. 2, lane 1).

Inheritance of the *GNA* gene in transgenic rice plants

Inheritance of the introduced *GNA* gene [from primary (R_0) transgenic plants] by the next (R_1) generation was examined by immunoblot analysis of selfed progeny derived from transgenic lines (Table 1). When the R_0 plants were crossed with untransformed plants, the *GNA* gene was inherited by

Table 1. R_1 inheritance of the *GNA* gene

Plant lines	No. of seedlings	GNA^+ (%)	GNA^-
GNA-3	30	22(73.3)	8
GNA-13	27	22(81.5)	5
GNA-63	28	21(75.0)	7
GNA-592	30	21(70.0)	9
GNA-614	25	23(92.0)	2

Expression of *GNA* gene was determined by western blot analysis. GNA^+ indicates R_1 progeny plants expressing the *GNA* gene. GNA^- indicates plant without the *GNA* gene.

R_1 lines GNA-3, GNA-63 and GNA-592 in a 1:1 segregation ratio, whereas a 3:1 segregation ratio was observed when the R_0 plants were selfed. This observation, along with the Southern blot analysis data, indicates that these lines carried a functional *GNA* gene at a single locus (data not shown). However, lines GNA-13 and GNA-614 contained three and five copies of the *GNA* gene, respectively.

Expression levels of GNA protein in transgenic rice plants

The presence of GNA protein in leaves of transgenic rice plants was analyzed by western blotting. Six independent R_1 (Fig. 3A) and R_2 (Fig. 3B) transgenic plants were assessed. A single band at 12 kDa, which co-migrated with the standard, was detected in plants transformed with *GNA* (Fig. 3, Lanes 3 to 8). Although two additional bands can be seen in lanes 7 and 8 in Fig. 3B, these bands are thought to be due to protein degradation. However, no 12 kDa band was observed in the protein extract from untransformed rice plants (Fig. 3, lane 2). The amount of GNA protein detected in the leaves of 8-week-old transgenic rice plants varied from plant to plant, generally ranging from 0.05% to 1.0% of total protein, with a maximum of 2.0%. Levels of GNA expression were estimated semi-quantitatively from the western blots. Variations in *GNA* gene expression were detected among both the R_1 and R_2 plants. Similar observations were made with transgenic rice plants expressing the GUS reporter gene under the control of the CaMV 35S promoter, in which variations in expression levels within a single genotype were frequently observed (data not shown).

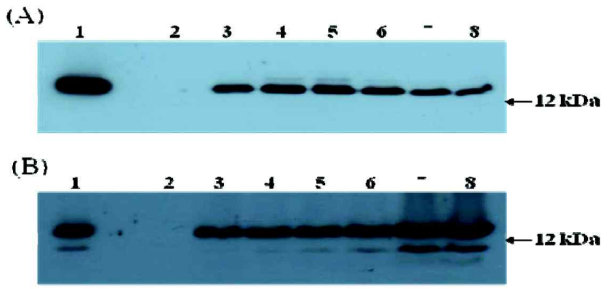

Fig. 3. Western blot analysis of transgenic rice plants. (A) Six R_1 lines and an untransformed rice plant. Lanes: 1, antigen; 2, untransformed plant; 3-8, proteins from lines GNA-3, GNA-4, GNA-13, GNA-63, GNA-592 and GNA-614, respectively. (B) Six R_2 progeny of line GNA-592. Lanes: 1, positive control; 2, untransformed plant; 3-8, R_2 progeny.

Effect of GNA on the survival of BPH

Bioassays of 2-month-old R_2 plants were performed using BPH. The number of BPH nymphs that fed on the transgenic rice plants declined significantly, beginning on the third day after infestation and continuing throughout the 8-day bioassay period (Fig. 4). For line GNA-592, the number of insects declined from 12 per plant (initial inoculum) to an average of six (50% of the controls) on Day 8. The BPH survival rate on line GNA-592 decreased by 40% compared with that on the control. This result corresponds with the levels of GNA protein in these transgenic rice plants.

The effect of BPH on transgenic rice plants

We investigated the effects of BPH on transgenic rice plants to determine whether *GNA* gene expression conferred protection against insect predation. To assess the effectiveness of the GNA protein in whole transgenic plants,

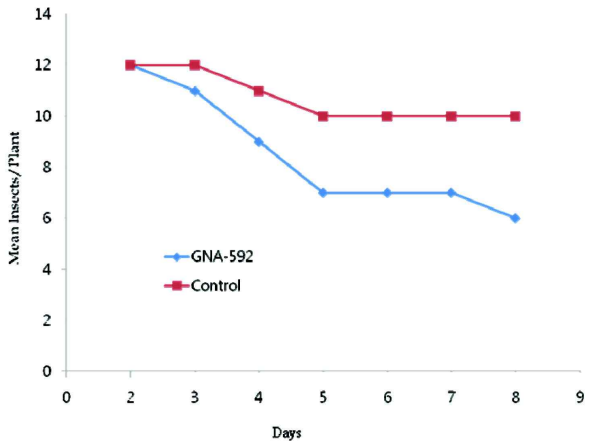

Fig. 4. Bioassays of BPH on transgenic rice plants expressing GNA. Twelve second-instar nymphs were released onto plants on Day 0. Transgenic plants (—◆—) were high GNA-expressing R_2 homozygous lines (GNA-592). Untransformed Nagdongbyeo plants were used as control (—■—).

Fig. 5. BPH resistance of an R_2 transgenic plant expressing *GNA*. A resistant transgenic plant showing healthy growth (right) and a susceptible control plant showing severe symptoms of BPH infestation (left) after 4 weeks of infestation.

2-month-old R_2 GNA-592 transgenic plants (which contained 2.0% GNA protein) and non-transgenic plants were infested with BPH for 4 weeks. Both transgenic and control rice plants were infested with second- and third-instar nymphs (100 per plant). Non-transgenic plants were severely damaged by BPH after 4 weeks (Fig. 5; left), whereas plants expressing GNA showed only minor leaf damage (Fig. 5; right). After 4 weeks, both egg production and the number of nymphs produced by the adults feeding on transgenic rice plants were reduced by approximately 6-fold compared with the control. The results of this bioassay provide further evidence that GNA can reduce insect attacks on rice, and that GNA has insecticidal activity against BPH. We hope to conduct further large-scale experiments under field conditions. As tissue-specific expression of a single *GNA* gene in transgenic plants induced insect resistance, we are currently investigating the use of other tissue-specific promoter (*RSs1*, which is a phloem-specific promoter) to further increase resistance to insect pests.

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초록 : Snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) 유전자 도입에 의한 벼멸구 저항성 형질전환 벼 개발

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유전자총(Particle bombardment) 방법에 의해 snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) 유전자가 도입된 해충저항성 형질전환 벼를 개발하였다. *GNA* 유전자가 도입된 많은 형질전환 식물체가 재분화되었으며, 형질전환 벼의 *GNA* 유전자의 integration, expression 및 inheritance는 Southern 및 western analysis분석 방법에 의해 증명되었다. 벼 genome 내 도입 유전자 수는 one 혹은 five copies이었다. 형질전환 R_1과 R_2 식물체의 GNA 단백질 발현 분석 결과, 함량은 총 단백질 중 0.01%부터 2.0%까지 포함하고 있었다. 형질전환 식물체 중 GNA 과발현 형질전환 벼를 이용한 생물 검정 결과, 대조구와 비교하여 벼의 주요 해충인 벼멸구(*Nilaparvata lugens* Stål)에 저항성을 나타내었다. 위의 실험 결과를 통해, 해충저항성 유전자인 *GNA*가 도입된 형질전환 벼는 해충의 증식을 억제하는데 이용될 수 있을 것이다.