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Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown planthopper

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Summary

Snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) has been shown previously to be toxic towards rice brown planthopper (*Nilaparvata lugens*; BPH) when administered in artificial diet. BPH feeds by phloem abstraction, and causes 'hopper burn', as well as being an important virus vector. To evaluate the potential of the *gna* gene to confer resistance towards BPH, transgenic rice (*Oryza sativa* L.) plants were produced, containing the *gna* gene in constructs where its expression was driven by a phloem-specific promoter (from the rice sucrose synthase *RSs1* gene) and by a constitutive promoter (from the maize ubiquitin *ubi1* gene). PCR and Southern analyses on DNA from these plants confirmed their transgenic status, and that the transgenes were transmitted to progeny after self-fertilization. Western blot analyses revealed expression of GNA at levels of up to 2.0% of total protein in some of the transgenic plants. GNA expression driven by the *RSs1* promoter was tissue-specific, as shown by immunohistochemical localization of the protein in the non-lignified vascular tissue of transgenic plants. Insect bioassays and feeding studies showed that GNA expressed in the transgenic rice plants decreased survival and overall fecundity (production of offspring) of the insects, retarded insect development, and had a deterrent effect on BPH feeding. *gna* is the first transgene to exhibit insecticidal activity towards sap-sucking insects in an important cereal crop plant.

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Introduction

Insect-resistant transgenic crop plants have been obtained through the introduction of *Bacillus thuringiensis* (Bt) crystal protein genes (*cry* genes) in several species (Estruch *et al*., 1997; Peferoen, 1992). Attempts have also been made to produce insect-resistant transgenic plants through the introduction and expression of foreign genes encoding plant defensive proteins, such as protease inhibitors or lectins, which showed anti-metabolic effects against certain insects (Boulter *et al*., 1990; Hilder *et al*., 1987). Transgenic plants which express Bt endotoxins or protease inhibitors have been useful for control of insects which feed by chewing plant tissues, such as species of the orders Lepidoptera and Coleoptera, but have not been shown to possess enhanced resistance towards sap-sucking insects belonging to the order Homoptera.

Two major pests of rice, the rice brown plant hopper (BPH) and the rice green leafhopper, are homopterans. They cause severe physiological damage to the rice plant, and act as vectors of the economically important rice tungro virus, grassy stunt virus and ragged stunt virus (Mochida *et al*., 1979; Saxena and Khan, 1989). Earlier studies (Powell *et al*., 1993), using bioassays based on an artificial diet feeding system, showed that plant lectins were toxic to these insects, and that snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) was the most toxic of the lectins tested, decreasing BPH survival by 50% at concentrations as low as 6 μM (Powell *et al*., 1995b). GNA is a tetrameric protein consisting of identical subunits of approximately 12 kDa, and shows specificity towards α-1,3- or 1,6-linked D-mannose residues in carbohydrates (van Damme *et al*., 1987). Although GNA is toxic to insects, it is non-toxic to mammals (Pusztai, 1991).

Results obtained with artificial diets suggest that a gene encoding GNA may be a good candidate to introduce into transgenic rice to provide resistance against rice brown planthopper and rice green leafhopper. The use of phloem-specific promoters to express an insecticidal protein in phloem tissue of transgenic rice plants would be especially useful for confering resistance to sap-sucking insects. The present paper describes the production of transgenic rice plants in which the phloem-specific rice sucrose synthase-1 gene promoter (*RSs1*; Shi *et al*., 1994) and the constitutive maize ubiquitin-1 promoter (*ubi1*) have been used to drive expression of *gna*. The effects of expression of GNA in transgenic rice on survival, development, fecundity and feeding of BPH are described.

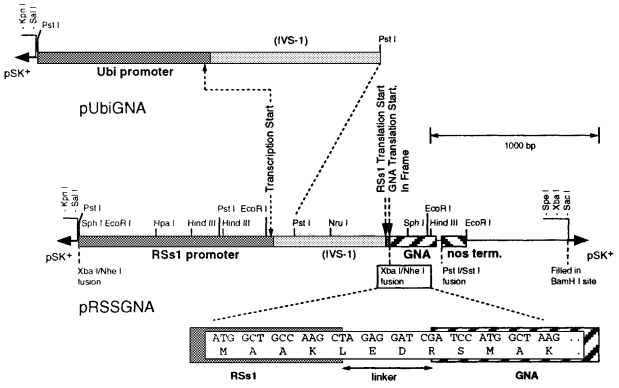

Figure 1. Structure and partial restriction maps of the plasmids pRSSGNA and pUbiGNA, showing the nucleotide sequence determined for the *RSs1*–GNA coding sequence fusion.

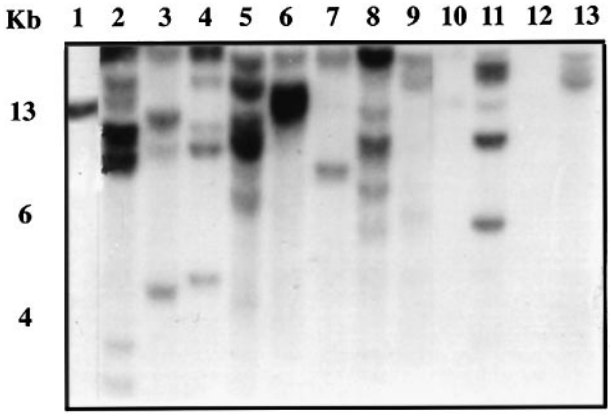

Figure 2. Southern blot analyses of genomic DNA from leaves of transgenic rice plants produced by particle bombardment of immature embryos. Genomic DNA (5 μg) was restricted with *Sac*I. The 480 bp GNA coding sequence amplified by PCR was used as the probe. Lane 1, one gene copy equivalent of plasmid pRSSGNA restricted with *Sac*I. Lanes 2–6, DNA from plants transformed with pRSSGNA; lanes 7–11 and 13, DNA from plants transformed with pUbiGNA; lane 12, DNA from control rice plant.

Results

Transformation and molecular analysis of transgenic rice plants

Transgenic rice plants produced by two different transformation technologies have been used.

Transgenic rice produced by electroporation of rice protoplasts

Rice protoplasts were co-transformed with two plasmids: pG35barB containing the *bar* gene driven by the CaMV 35S promoter, and pRSSGNA containing the GNA gene driven by the rice sucrose synthase promoter (Figure 1). Plants were regenerated from the selected phophinothricin-resistant calli. Putative transgenic plants were tested by topical application of the herbicide Basta; a treated portion of untransformed plant leaf tissue showed complete damage, whereas transgenic plants exhibited less or very little damage.

Transgenic rice produced by particle bombardment of immature rice embryos

Transgenic rice plants were produced by co-transformation with plasmids pWRG 1515 (containing genes *hpt* and *gusA* encoding selectable and screenable markers, respectively) and pRSSGNA or pUbiGNA (containing the maize ubiquitin promoter fused to the GNA coding sequence; Figure 1), by particle bombardment of immature embryos (Christou *et al.*, 1991). Transformants were selected by exposure to hygromycin.

Molecular analysis of transformants

DNA samples from Basta-tolerant and control plants produced by protoplast transformation were tested for the presence of the GNA coding sequence by PCR, which gave the expected 480 bp fragment in positive plants (results not shown). Southern blots of undigested DNA from PCR-positive plants showed hybridization with the *bar* and GNA probes in the high-molecular-weight regions indicating that the *bar* and GNA genes had integrated into the rice genome (results not shown). When genomic DNA from positive transformants was analysed by Southern blotting after digestion with *SacI* (Figure 1), similar results were obtained for plants produced by both methods. Bands of both higher and lower molecular weights than the plasmid (13 kb) were detected by a GNA coding sequence probe (Figure 2), at intensities significantly higher than the one gene copy equivalent (e.g. Figure 2, lane 2), showing that multiple copies of the plasmid had been integrated into the rice genome.

Expression and inheritance of GNA and selectable marker genes

Western blot analyses of leaf extracts from transgenic rice plants derived from both pRSSGNA and pUbiGNA showed the presence of a polypeptide of approximately 12 kDa, corresponding to the GNA standard, when probed with anti-GNA antibodies (Figure 3); a minor band of lower M_r was also present in some samples. More than 50% of plants derived from particle bombardment expressed GNA at high levels. Levels of GNA expression were estimated semi-quantitatively from the Western blots; leaf tissue from positive plants derived from pRSS1GNA contained GNA in the range 0.01–0.25% of total soluble protein, whereas plants derived from pUbiGNA contained GNA at up to 2.0% of total soluble protein.

R_1 progeny plants from protoplast-derived primary trans-

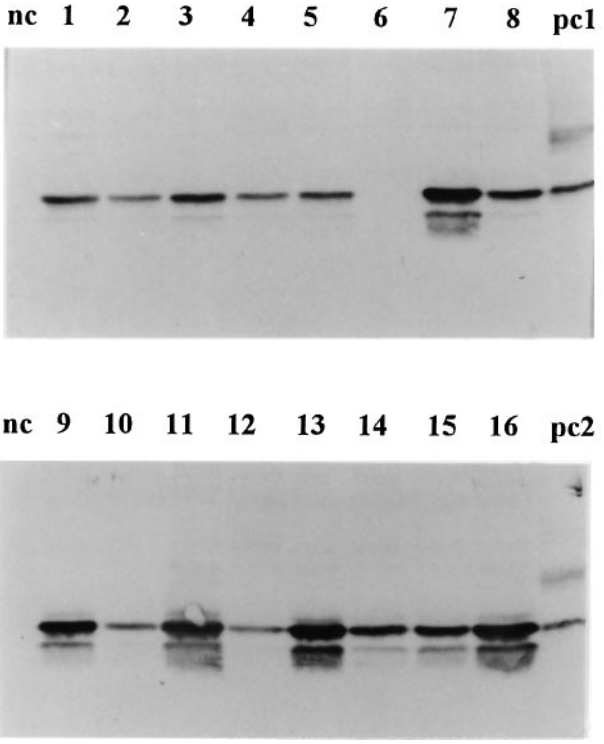

Figure 3. Western blot analyses of protein extracts from leaves of transgenic rice plants (R_0).
Equal amounts of protein (5 μg) were loaded in each lane. Lane 'nc', extracts from non-transformed control plant; lane 'pc', GNA standards (50 ng). Lanes 1–8, extracts from plants transformed with pRSSGNA; lanes 9–16, extracts from plants transformed with pUbiGNA.

formants GNA 2 and GNA 4 were analysed for resistance to Basta, for the presence of the GNA coding sequence by PCR, and for GNA expression by Western blotting. Resistance to Basta was observed to segregate in these progeny plants in a ratio consistent with a 3:1 segregation (Table 1), suggesting integration of the transgene(s) at a single locus. The GNA coding sequence was found in 21 out of 28 progeny of GNA 2. However, Western blot analyses of the same plants only detected expression of GNA in 15 of the 21 plants determined as positive by PCR. Further analysis of plants showed a consistent under-representation of GNA expression (compared to the predicted ratio of 3:1 expressers:non-expressers) detected on Western blots. Analysis for inheritance of GNA protein was also carried out in R_1 and R_2 progeny plants derived from selected primary transformants produced by particle bombardment. This inheritance was consistent with integration of the foreign gene at a single locus, and under-representation of GNA-expressing plants was not observed (data not presented).

Localization of GNA expressed in transgenic rice plants

GNA expression was localized in glutaraldehyde-fixed sections of transgenic plants derived from pRSSGNA by reaction with anti-GNA antibodies (Figure 4). In agreement with the predicted pattern of expression for the *RSs1* promoter, GNA was detected in phloem cells and other non-lignified vascular tissue, with high levels present in the phloem companion cells. Expression of GNA was also detectable in epidermal cells of leaf sections, but mesophylll cells were not stained. Sections taken from different leaf blades gave identical results. GNA expression in transgenic plants derived from pUbiGNA showed no apparent tissue specificity (results not presented).

Effects of GNA expression in transgenic rice on brown planthopper

Survival

BPH survival was monitored from late first-instar nymphs to adults at two-day intervals, on progeny plants produced by selfing primary transformants, which had been derived from transformation with pRSSGNA and pUbiGNA. GNA expression was assayed in the plants used, which were divided into three groups for each treatment: controls, transgenic progeny not expressing GNA (GNA–), and transgenic progeny expressing GNA (GNA+). Survival declined from ten insects per plant (initial inoculum) to approximately seven insects per plant over a 22-day bioassay period on the control plants, and the survival curves for the GNA– transgenic progeny plants did not differ significantly from controls. However, BPH on both types of GNA+ transgenic progeny plants showed lower survival than the controls throughout the assay period, with differences significant ($P < 0.05$) after day 4 (Figure 5). The difference between control and GNA+ increased consistently throughout the assay, so that by day 20, BPH survival on GNA+ plants derived from pRSSGNA and pUbiGNA was approximately 62% and 59% of controls, respectively.

Development and fecundity

The effects of GNA expression on insect development were measured by releasing early first-instar nymphs onto GNA-expressing progeny plants derived from transformation with pRSSGNA. In broad agreement with results above, BPH survival on the transgenic plants was reduced to a mean of 7.6 insects/plant compared to a mean of 11.6 insects/plant on control plants after 13 days (initial inoculum 25 insects/plant), although this difference was not statistically significant ($P \sim 10\%$) due to the small number of plants tested (Figure 6a). Insects on the transgenic plants were however significantly retarded in development compared with controls, with only 0.8 insects per plant (11% of survivors) reaching adulthood after 13 days on the transgenic plants, compared with 6.4 insects per plant (55% of survivors) on controls (significantly different at $P < 0.01$). The poor development of BPH nymphs on the transgenic

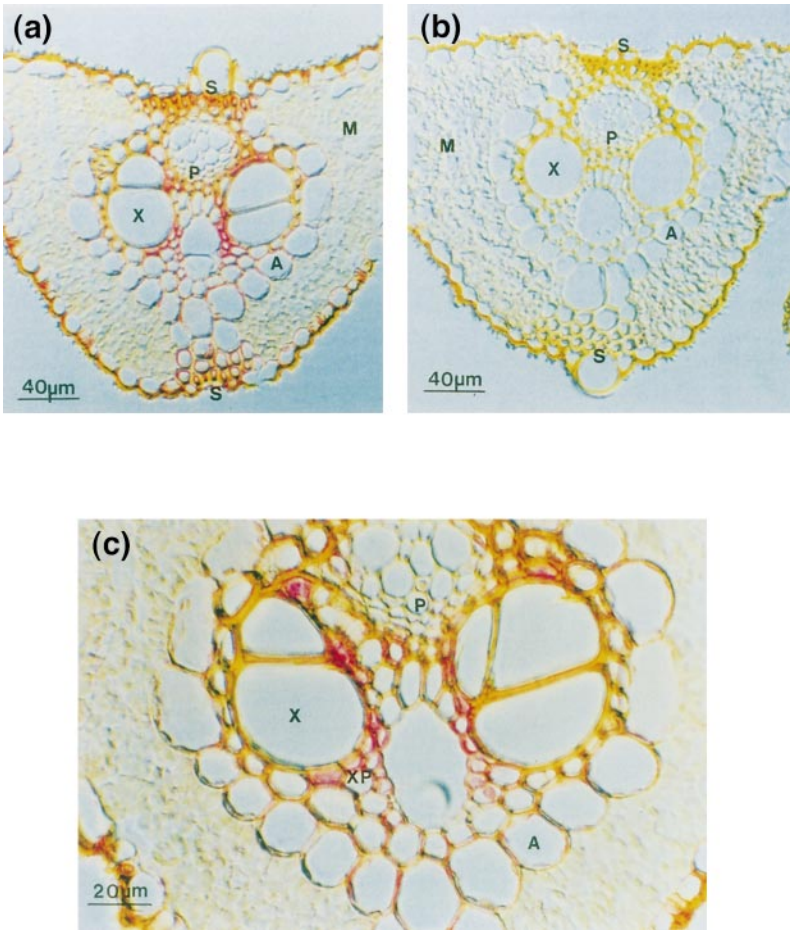

Figure 4. Immunohistochemical localization of snowdrop lectin (GNA) in transgenic rice containing the *RSs1*–GNA construct (transformant GNA4 from protoplast transformation experiments).
The presence of GNA is shown by the insoluble red product of the reaction between Fast Red and the hydrolysis product of naphthol phosphate.
(a) Cross-section of leaf blade showing localization of GNA in the vascular tissues and epidermis of the plant.
(b) Control section to (a) incubated in pre-immune serum.
(c) Section similar to (a), at higher magnification, showing localization of GNA within the vascular region; GNA is present in the phloem companion cells and other non-lignified vascular cells.
A, accessory cells; M, mesophyll; P, phloem; S, schlerenchyma; XP, non-lignified vascular tissues (xylem parenchyma); X, xylem vessels.

Table 1. Transmission of *bar* gene to R_1 progeny of parents GNA 2 and GNA 4

Parent	Number of progeny plants analysed	Number of Basta-resistant plants	Number of Basta-susceptible plants	χ^2 (3:1)
GNA 2	67	48	19	0.402[a]
GNA 4	5	4	1	0.067[a]

[a]The difference from the expected 3:1 segregation is not significant at $P < 0.05$.

plants was also reflected in a 52% decrease in the mean insect biomass per plant (approximately 20% decrease on a per insect basis) after 13 days (significant at $P < 0.05$; Figure 6a).

Effects of GNA on BPH fecundity were assessed by measuring total nymph production by adult insects on the transgenic rice plants used for survival assays. Over a 40-day assay period, the number of BPH nymphs produced per plant was reduced by approximately 30% (significant at $P < 0.01$) in GNA+ transgenic progeny plants derived from transformation with pRSSGNA, and by approximately 45% (significant at $P < 0.001$) in GNA– transgenic progeny plants derived from pUbiGNA (Figure 6b), compared to controls. Nymph production by BPH on GNA– progeny plants did not differ significantly from controls.

Feeding

Transgenic rice plants (GNA+) derived from transformation with pRSSGNA also had a significant effect on BPH feeding, as estimated by a semi-quantitative assay of honeydew (liquid excreta) production. Late first-instar BPH nymphs (pre-starved) were transferred to plants, and feeding was estimated over a 48 h period. A decrease in estimated honeydew production on the transgenic plants of 78% (significant at $P < 0.01$) compared to the control plants was

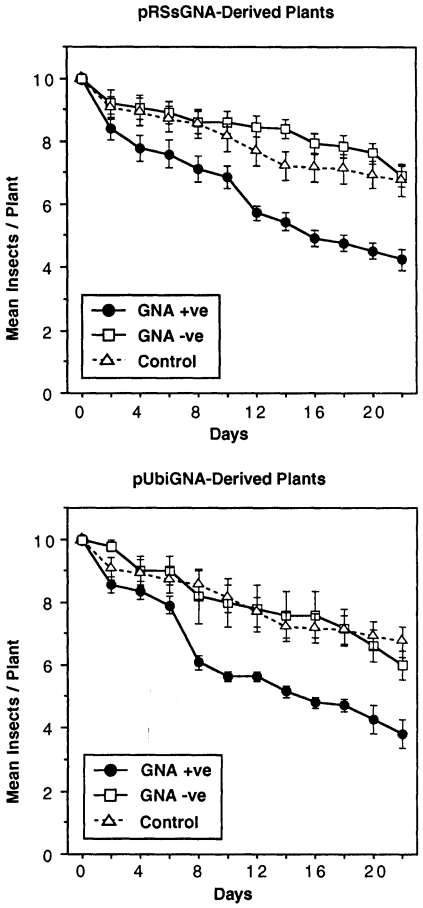

Figure 5. Survival of BPH on transgenic rice plants expressing GNA.
Ten first-instar nymphs were inoculated onto each plant at day 0. Transgenic plants are progeny derived from transformants produced by particle bombardment, and are divided into GNA+ (expressers) and GNA– (non-expressers) for plants containing the *RSs1*–GNA construct (phloem-specific GNA expression), and the *Ubi*–GNA construct (constitutive GNA expression). Points and bars show means ± SE (control, $n = 16$; *RSs1*–GNA+, $n = 15$; *RSs1*–GNA–, $n = 13$; *Ubi*–GNA+, $n = 11$; *Ubi*–GNA–, $n = 5$); differences between GNA+ plants and controls are significant ($P < 0.05$) after day 4 (ANOVA).

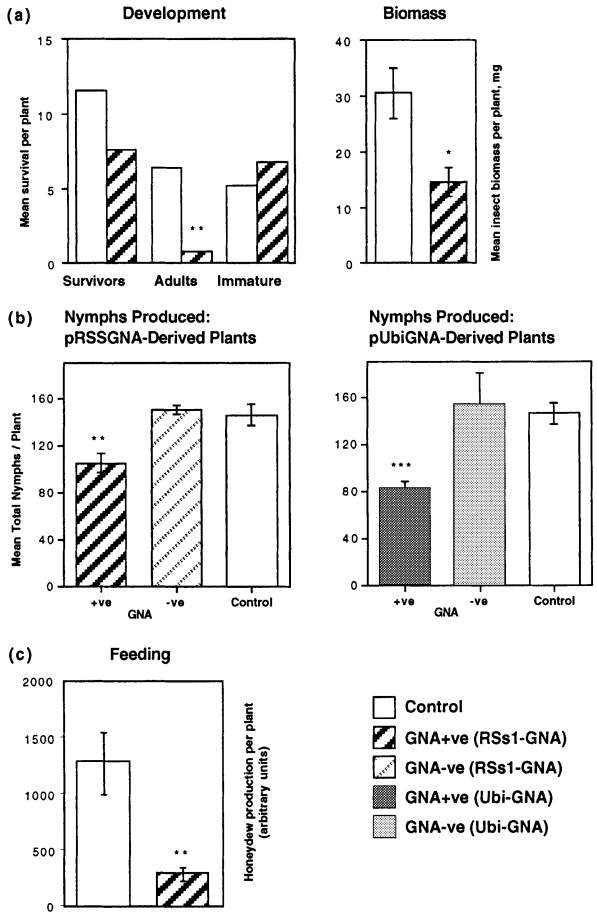

Figure 6. Effects of transgenic rice plants expressing GNA on development, overall fecundity and feeding of BPH.
(a) Effects on development: 25 neonate first-instar BPH nymphs were released onto each plant at day 0. Transgenic plants are progeny derived from a transformant produced by protoplast electroporation, containing the *RSs1*–GNA construct (phloem-specific GNA expression) and were selected for GNA expression. After 13 days, insect survival, biomass and development were determined on a per plant basis; differences in survival were assessed by a Mann–Whitney *U* test, and differences in biomass by an unpaired *t* test. $^{*}P < 0.05$; $^{**}P < 0.01$ ($n = 5$).
(b) Effects on overall fecundity. The total number of nymphs produced per plant from adult BPH on rice plants as in Figure 5 was counted from start to end of nymph emergence. Differences in nymph production were assessed by unpaired *t* test; $^{**}P < 0.01$; $^{***}P < 0.001$.
(c) Effects on feeding. Honeydew excretion by BPH nymphs feeding on control rice plants and transgenic rice plants (*RSs1*–GNA, as in (a)) was estimated semi-quantitatively on a per plant basis over a 48 h period. Each plant was inoculated with 25 late first-instar BPH nymphs. The difference between control and experimental is significant (**) at $P < 0.01$ (unpaired *t* test; $n = 5$).

observed, as shown in Figure 6(c). No significant difference in mortality of the insects was observed during the feeding period.

Discussion

Transgenic rice plants co-transformed with the *bar* and *gna* genes, or *hpt*, *gusA* and *gna* genes were regenerated from protoplast-derived calli and from bombarded immature embryos. In both cases, multiple copies of the desired transgenes were introduced (Figure 2), although the segregation analyses of progeny plants showed that the integration events had occurred at a single locus. Analysis of the progeny of primary transformant GNA 2 (derived from protoplast electroporation with pRSSGNA) showed a 3:1 segregation pattern for both herbicide resistance and the presence of the GNA coding sequence: about 71% of the plants that contained the GNA gene produced detectable levels of protein. Some plants that scored negative for GNA expression may have produced the protein but at levels below the limits of detectability. It is also possible that 'gene silencing' may have occurred, as has been observed in progeny of other transgenic plants (Matzke *et al.*, 1996). Similar results were obtained with GNA-

expressing plants derived from particle bombardment, although the higher number of transformants produced allowed selection of lines with high and stable GNA expression, and Mendelian inheritance of the gene and the encoded protein was observed in R_1 and R_2 generations. Further work will be necessary to assess the stability of expression of GNA in transgenic rice lines over extended generations. The relatively lower expression levels of GNA in plants derived from pRSSGNA compared to the levels in plants containing the *ubi1* promoter is due to the low abundance of phloem cells in the plant, and does not necessarily reflect an inherently low activity of this promoter (Shi *et al.*, 1994).

The immunolocalization of GNA in the leaves of primary transformant plant GNA 4 (derived from protoplast electroporation with pRSSGNA) and in plants derived from particle bombardment (results not presented) confirmed the tissue-specific expression determined by the *RSs1* promoter. Highest levels of GNA were seen in phloem companion cells, but the protein was also present in the sieve tubes and other non-lignified vascular tissue and at lower levels in epidermal cells. The comparatively low level of staining for GNA observed in sieve tubes may be a consequence of technical difficulties in fixing the contents of these cells. GNA can be very readily detected in tissue blots of the cut ends of leaves (sectioned across the blade) from plants transformed with pRSSGNA, and is localized to vascular regions, suggesting that the protein is present in phloem exudate (results not presented). The pattern of expression in non-lignified vascular tissue is similar to that observed when the *RSs1*–GNA construct was expressed in transgenic tobacco (Shi *et al.*, 1994). Although other tissues of the plant were not examined, expression in the phloem throughout the entire plant would be expected on the basis of previous localization experiments of sucrose synthase in monocots (Nolte and Koch, 1993). Sucrose synthase has also been localized in the epidermal cells of cotton ovules (Nolte *et al.*, 1995), and thus the observation that GNA is present in the epidermis of transgenic rice leaves is not inconsistent.

The insect bioassay results presented here can be compared to those observed when GNA was delivered to aphids (also homopteran pests of plants) via transgenic plants (Down *et al.*, 1996; Gatehouse *et al.*, 1996). GNA expression had no effect on aphid survival, but slightly retarded development and decreased fecundity (time to and rate of production of parthenogenetic offspring, respectively). Effects of GNA expression on BPH are clearly apparent as a decrease in survival (Figure 5), although there is also good evidence that growth and development from nymph to adult is retarded (Figure 6a). Although GNA expression has a significant deleterious effect on the overall fecundity of BPH (Figure 6b), some or all of this effect could be due to lower survival of the insects rather than decreased egg production and/or lower viability of eggs. BPH only reproduces sexually, and thus direct comparison with parthenogenetic aphids is not appropriate. Previous assays with artificial diets have shown that GNA has significant effects on BPH survival, when delivered at levels above approximately 4 μM (Powell *et al.*, 1995b), but insect fecundity could not be estimated using the artificial diet system. Further experiments will be required to determine the effects of GNA on the fecundity of BPH on a per insect basis. Despite the different levels of GNA as a proportion of total protein, plants derived from pRSSGNA and pUbiGNA gave similar results in the insect bioassays, suggesting that the phloem-specific promoter was effective in delivering GNA to the insects, although the concentrations of GNA encountered by the insects when feeding on transgenic plants were not directly measured. Plant transformation *per se* had no effect on any of the insect parameters measured.

A depression in BPH feeding, as measured by honeydew production, has been observed when the protein is fed in artificial diet (Powell *et al.*, 1995a), and a similar result on GNA+ transgenic plants was indicated in these bioassays (Figure 6c). Studies of BPH feeding patterns by the electrical penetration graph method (unpublished results) have shown that GNA strongly inhibits active ingestion of diet *in vitro*, but does not deter the insect from probing behaviour. If this is duplicated *in vivo*, transgenic rice may still be vulnerable to viral transmission by probing activity.

In conclusion, this paper presents results which show that transgenic rice plants can be partially protected against brown planthopper by expression of the insecticidal lectin GNA in their tissues. The introduction of this novel resistance gene into the germplasm of rice makes the trait available for conventional rice breeding programmes aimed at resistance against the brown planthopper.

Experimental procedures

Plasmid constructs and protoplast transformation

The preparation of a gene construct containing the GNA coding sequence from pLECGNA2 (Van Damme *et al.*, 1991) and the promoter region from the rice sucrose synthase gene *RSs1* (Wang *et al.*, 1992) has been described previously (Shi *et al.*, 1994). The construct contains a translational fusion between the first five amino acids of the *RSs1* coding sequence and the complete GNA pre-protein sequence; due to the presence of linker fragments, several additional amino acids are also introduced (see Figure 1). The *RSs1*–GNA construct was excised from the binary vector (pBI101.2) and ligated into the plasmid vector pSK+ to give the recombinant plasmid used for rice transformation, pRSSGNA (Figure 1).

The isolation of protoplasts from rice (cv. Radon and Nortai) suspension cultures was carried out as described by Lee *et al.* (1989). Protoplasts at a density of 1×10^7 per ml were mixed with 50 μg (1 μg μl^{-1}) each of plasmids pRSSGNA and pG35barB

(containing the construct CaMV35S-*bar-nos*-poly(A)+; Rathore *et al.*, 1993) and electroporated (Rao *et al.*, 1995). The protoplast density was adjusted to 5.0×10^6 per ml and 300 μl aliquots were plated on a Millipore filter located on top of feeder cells and cultured (Peng *et al.*, 1990). After 3–4 weeks' growth on IR-52 nurse cells, the microcalli were transferred to Linsmaier and Skoog (1965) medium supplemented with 0.5 μg ml^{-1} 2,4-D and 6.0 or 8.0 μg ml^{-1} phosphinothricin (PPT) for selection of transformed calli. The actively growing PPT-resistant calli were selected and transferred to proliferation medium (Rathore *et al.*, 1993). Putatively transformed calli were regenerated into plants and were transferred to sterile soil and grown to maturity in the growth chamber.

Transformation of rice by particle bombardment of immature embryos and recovery of transgenic plants was carried out as described by Christou *et al.* (1991). Three diverse indica rice genotypes, ASD 16, M5 and M12, and one elite japonica genotype, FX 92, were used in this study. Plasmids pRSSGNA or pUbiGNA were co-transformed with pWRG1515 (containing the *gusA* and *hpt* genes, driven by the CaMV 35S promoter); transformants were selected on media containing hygromycin (50 μg ml^{-1}). A total of 200 independent transgenic plants representing approximatley equal numbers derived from pRSSGNA and pUBiGNA were obtained.

PCR and Southern blot analyses

Regenerated plants were tested for their tolerance to the herbicide Basta (0.25%) as described by Rathore *et al.* (1993). Isolation of DNA from leaf tissue from Basta-resistant plants along with control plants was carried out according to the method of McCouch *et al.* (1988), or by the CTAB extraction method (Creissen and Mullineaux, 1995). PCR analysis for detection of the GNA gene was carried out using the primers 5′-CGGATCCATGGCTAAGGCAAGTCTCCTC-3′ and 5′-CGGTACCTCATTACTTTGCCGTCACAAG-3′ that were part of the coding region of the GNA gene. PCR products were analysed by gel electrophoresis on 1.0% agarose gels. Primers amplifying the coding sequence of the rice oryzacystatin *Ozc-1* gene were used as a positive control for PCR.

Procedures for restriction enzyme digestion, electrophoresis and Southern blot analyses were according to Sambrook *et al.* (1989) or the manufacturer's recommendations. Southern analyses were performed with 5 or 12 μg of genomic DNA digested with *Sac*I, *Kpn*I or *Xba*I. The PCR-amplified product (480 bp), which represents the complete coding sequence of the GNA, from plasmid pRSSGNA, or a *Hin*dIII fragment containing the GNA coding sequence, and the *Bgl*II–*Bam*HI fragment (563 bp) of pG35barB, which represents most of the *bar* coding sequence, were radiolabelled with [α-^{32}P]dCTP and used as probes. Filters were washed to a stringency of 0.2× SSC, 0.5% SDS at 65°C, and autoradiographed.

Western blot analyses

Samples of leaf tissue were homogenized in 50 mM Tris–HCl buffer, pH 9.0. The extract was centrifuged at 2000 *g* for 10 min at 4°C, and the supernatant was collected. Protein samples (5 μg) were subjected to SDS–PAGE according to Laemmli (1970). Following electrophoresis, proteins were transferred onto nitrocellulose membranes (Amersham) by electroblotting (Towbin *et al.*, 1979). After protein transfer, filters were soaked in 2% periodic acid (10 min), rinsed, and blocked by incubation in 10% PBS solution containing 10% non-fat milk, 0.1% Tween-20 for 2 h at room temperature. The filter was probed with polyclonal rabbit anti-GNA serum (1:10 000 dilution) and goat anti-rabbit IgG horseradish peroxide conjugate (Bio-Rad) as a secondary antibody (1:10 000 dilution). Incubation and washing procedures were according to standard protocols (Amersham). Bound secondary antibodies were detected by enhanced chemiluminescence (ECL) as described in the manufacturer's protocols (Amersham).

Immunohistochemical localization of GNA

Excised rice leaves were cut into pieces about 3 mm wide and immediately placed into fixative solution (4% paraformaldehyde, 1.25% gluteraldehyde in PBS). The immunohistochemical detection of GNA was carried out as described by Harris (1994), using primary antiserum (anti-GNA, see above) diluted 1:200 in Tris-buffered saline (TBS) containing 0.01% v/v Tween-20, and alkaline phosphatase-conjugated secondary antibody diluted 1:200 in blocking solution. The sections were washed for 5 min in TBS, and then washed twice for 5 min each in distilled water.

Sections were then incubated in substrate solution (prepared by adding 100 μl of Fast Red stock (Sigma Chemical Co.; 51.4 mg ml^{-1} in 70% DMF) and 100 μl naphthol A-S-MX phosphate stock (Sigma Chemical Co.; 20.8 mg ml^{-1} in 100% DMF) to 5 ml of developing buffer (0.1 M Tris–HCl, pH 9.6, 0.1 M NaCl, 0.1 M $MgCl_2$) at 37°C in the dark until the colour developed. Sections were then washed twice for 5 min each in distilled water and then mounted in citifluor and viewed under bright field or DIC illumination.

Insect bioassays

Rice brown planthopper (*Nilaparvata lugens*), originally obtained from Rhone-Poulenc Agriculture Ltd, Essex, UK, was maintained on 40–60-day-old rice plants (var. TN1) in a controlled environment chamber (25 ± 2°C, 70–80% relative humidity, 16 h light/8 h dark). Plants used in the bioassays were grown in peat-based compost standing in approximately 1 cm water, under the same conditions; at the start of the bioassays plants were 25–30 cm tall, approximately 6 weeks post germination.

To carry out insect bioassays, each plant was confined in an insect-proof fine-mesh nylon cage so that insect transfer between plants was not possible. Experiments where insect parameters were measured only at the conclusion of the assay were carried out without further confinement of the insects. Twenty-five late first-instar BPH nymphs were released onto each of the plants, and the experiment was continued for 13 days. Survival, stadium and total insect biomass were determined. For the long-term survival experiments, insects were further confined in a clear plastic container fitting around the stem of the plant, and sealed with nylon mesh. Ten first-instar BPH nymphs were introduced into each container, and survival was monitored at 2-day intervals for 22 days. To determine overall fecundity, the adult BPH (confined as for long-term survival experiments) were allowed to mate at random, and total nymphs produced from eggs laid were counted after a 40-day interval (by which time no new nymphs were emerging).

Insect feeding was estimated by measurements of the production of liquid excreta (honeydew) by insects. Twenty-five late first-instar BPH nymphs per plant (pre-starved for 1 h) were released into feeding chambers of the type developed at IRRI (Sogawa and Pathak, 1970), which had been fitted to transgenic and control rice plants. Honeydew was collected onto a filter paper at the bottom of the chamber for 48 h. The filter papers were removed and air-dried, and then sprayed with 0.1% ninhydrin solution in acetone.

After development for 5 min at 100°C, honeydew deposits were visible as purple spots. The area of the spots was measured using millimetre graph paper as a semi-quantitative estimate of the amount of honeydew produced (Powell *et al.*, 1995a).

Statistical analysis of the data was carried out using Statview version 4.5 (Abacus Concepts, California, USA) running on Apple Macintosh computers; *t* tests, ANOVA analysis or Mann–Whitney *U* tests were used to assess the significance of differences between treatments, as appropriate.

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