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Identification of receptors responsible for binding of the mannose specific lectin to the gut epithelial membrane of the target insects

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The sap-sucking homopteran insects, commonly known as aphids and leafhoppers are responsible for a huge amount of lost productivity of mustard, chickpea, cabbage, rice and many other important crops. Due to their unique feeding habits and ability to build up a huge population in a very short time, they are very difficult to control. The objective of the ongoing program is to develop insect-resistant crop species through genetic engineering techniques to combat the yield losses, which necessitates the identification of appropriate control elements. In this direction, mannose-binding 25 kDa lectins have been purified from leaves of garlic, *Diffenbachia sequina* and tubers of *Colocasia esculanta.* The purified lectins have been analyzed in SDS-PAGE. The effectiveness of these lectins against chickpea aphids, mustard aphids and green leaf hoppers of rice have been tested. The LC_{50} value of each lectin against different insects had been monitored [1,2]. Through immunolocalization analysis, the binding of the lectin had been demonstrated at the epithelial membrane of the midgut of the lectin-treated insects [1]. Receptor proteins of brush border membrane vesicle (BBMV) of the target insects, responsible for binding of the lectin to the midgut of the epithelial layer have been purified and analyzed through ligand assay. Biochemical studies have been undertaken to investigate the lectin-receptor interaction at molecular level.
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Introduction

Homopteran insects cause severe damage to crop plants like rice, mustards, peas, cabbages, and many others. Among them the important ones are, *Nephotettix sp.* (Green Leaf Hopper of rice), *Lipaphis erysimi* (mustard aphid), and *Aphis craccivora* (chickpea aphid). They not only destroy the plants by sucking on the plant sap with the help of their novel feeding apparatus but also transmit various disease-causing viruses into the plant, acting as vectors while feeding on the plant. These insects are insensitive to Bt toxin. Moreover, due to their unique feeding habits and fast multiplicity, they are difficult to control by applying insecticides. However, genetic engineering technology with a potent control agent has been thought to be an alternative solution, which necessitates the identification of an appropriate control agent against the above mentioned three target homopteran pests. For the last few years, different plant lectins have earned importance for being able to control the growth and multiplication of various insect pests [3–10]. In line with this we have isolated mannose-binding lectins from leaves of *Allium sativum*, *Diffenbachia sequina*, and tubers of *Colocasia esculanta* [2]. To judge the efficacy of all three lectins against the above mentioned three target pests, insect bioassay has been conducted in artificial diets supplemented with different doses of lectins. The LC_{50} values of individual lectins have been estimated by statistical analysis of the insect mortality data, obtained. Having the idea from our earlier report about the binding of the *Allium sativum* leaf lectin (ASAL) at the inner epithelial layer of the lectin treated red cotton bug insect, the same immunological approach has been taken to demonstrate the binding of ASAL onto the gut epithelial membrane of *Aphis craccivora*, the most common and serious pest of pulse crops, namely chickpea and pigeonpea. Subsequently, the brush border membrane vesicle (BBMV) proteins from all three tested insects were purified, and, through ligand blot analyses, the respective receptor proteins having binding specificity to ASAL have been identified and characterized.

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Materials and methods

Fresh garlic leaves have been collected from the plants grown in the institutional experimental farm. Leaves of *Diffenbachia*

and tubers of *Colocasia* have been collected from local gardens. Nymph and adult aphids of mustard (*Lipaphis erysimi*), chickpea (*Aphis craccivora*), and green leaf hopper of rice Nymph and adult aphids of mustard (*Lipaphis erysimi*), chickpea (*Aphis craccivora*), and green leaf hoppers of rice (*Nephotettix sp.*) have also been collected from the institutional farm.

Extraction and purification of lectin through affinity chromatography

Twenty grams of fresh leaf tissue of *Allium sativum* was homogenised in a shearing blender and the lectin (ASAL) was further purified initially through affinity matrix (mannose-agarose) and subsequently purified through DEAE Sephacel column with 20mM Sodium Acetate, pH 5.0 [1]. Similarly the lectins of *Diffenbachia* leaf (DEA) and *Colocasia* tuber (CEA) were purified as described in earlier reports [2].

Characterization of ASAL, DEA, and CEA through Agglutination assays

All three lectins were allowed to agglutinate rabbit erythrocytes as described earlier [1,2].

SDS-PAGE analysis

All Lectin preparations after purification were analyzed in 15% SDS-PAGE according to Laemmli [11].

Insect bioassay in artificial diet

Keeping the fact in mind that the sucking pests feed exclusively on the phloem saps of plants, bioassay has been set up on an artificial diet, the composition of which was close to natural foodstuff present in the phloem sap. The liquid diet has been formulated with some modifications [1,2] from the original description by Dadd and Mittler [12]. Forty-five 2nd instar nymphs of *Aphis craccivora* were incubated for each set in liquid diet of 200 μl supplemented with the lectins ASAL, CEA, and DEA (5, 10, 15, 20 μg/ml each). Data on survival of the insects were collected at every 24 h. Similar Bioassay have been conducted with 5th instar nymphs of *Nephotettix sp.* with different concentration of ASAL. The LC_{50} value of each toxin corresponding to each insect had been determined by statistical probit analysis [13].

Immunolocalization of ASAL in Aphis craccivora midgut

Insects of set A (maintained in artificial diet only) and set B (maintained in artificial diet supplemented with 2 nanomolar ASAL) were narcotized within a petri dish by throwing in chloroform-soaked cotton. The appendages and the wings were excised and the whole gut of the insects dissected out and tentatively stored in insect ringer solution (0.65% NaCl, 0.25% KCl, 0.03% $CaCl_2$) to maintain tonicity of the membranes. The cryomicrotome was used at a chamber temperature of -20°C and blade temperature of $-20^{\circ} \pm 1^{\circ}$C. On an average, 20 sections of 5μ thickness were collected on a single slide from each set A and B and allowed to slowly return to room temperature. After the embedding medium surrounding the thin sections had liquefied, the slides were gradually dipped into sufficient volume of ringer solution in a petri dish and were layered on the Meyer's adhesive. Few sections from set A were taken aside, incubated in 2 nanomol ASAL in PBS and washed with PBS, prior to further processing and designated as set C. At this point, all the three sets were incubated in 3% non-fat milk (Sigma Immunochemicals) for 2 h at 37°C to block any non-specific IgG binding sites. Anti-ASAL polyclonal antibody, diluted 1:1000 in PBS, was added to the slides and incubated at 37°C for 2 h. Thorough washing was carried out with PBST (PBS with 0.02% Tween-20, Sigma) and further incubated with Goat anti-rabbit IgG-horseradish peroxidase conjugate (Sigma Immunochemicals) for 2 h at 37°C. Thorough washing was repeated with PBST with three changes of 15 minutes each. Color was developed by incubating with 3,3′-diaminobenzidine hydrochloride (DAB, Gibco BRL) and 0.08% H_2O_2 [Merck, Germany] in PBS. Slides were incubated with this substrate in the dark until coloration was apparent.

Isolation of brush border membrane vesicle (BBMV)

For total BBMV isolation, approximately 1000 aphids and 1000 green leafhoppers were dissected under a simple microscope, and their midguts excised. Until further processing, the excised midguts were stored at room temperature in 'insect ringer solution' (0.65% NaCl, 0.25% KCl, 0.03% $CaCl_2$). The isolated mid guts were homogenized in a hand-held Potter homogenizer in minimum volume of the 'isolation buffer' (450 mM Mannitol, 5 mM EGTA in PBS). Total BBMV was isolated according to Bandyopadhyay *et al.* [1] using the principle of Magnesium Chloride sequential precipitation of luminal membranes.

Purification of BBMV receptor protein

The major receptor proteins, identified from the ligand blot analysis, were purified from preparative SDS-PAGE. Approximately 100 ml of the insect BBMV protein extract was loaded onto the preparative well of a 12% SDS polyacrylamide gel along with protein molecular weight standards in the adjoining small well. Electrophoresis was carried out in Bio-Rad mini protean III electrophoresis module in accordance to the instructions of the manufacturer. On completion of the run, the marker well, along with 3–4 mm of the preparative well, was excised and put up for coomassie brilliant blue staining. The rest of the preparative gel was stored at 4°C. On complete staining and destaining of the strip, it was aligned with the stored unstained gel and the receptor band excised from the latter. The receptor protein was electroeluted from the gel pieces using the ATTO electro-elution apparatus (ATTO Corporation, Japan). The eluted sample was dialyzed against 50 mM Ammonium

Bicarbonate buffer, pH 8.0; the resulting SDS-free protein sample was lyophilized to dryness and subsequently analyzed with a ligand blot experiment as described earlier.

Characterisation of the BBMV receptors

The first dimension Isoelectric Focusing (IEF) was carried out in Bio-Rad Mini Protean II Isoelectric focusing module with carrier ampholytes according to the protocol given by the manufacturer. The protein samples (approximately 5 mg each) were mixed with an equal volume of IEF sample buffer with 1:4 pH 3–10 and 5–8 carrier ampholytes and run in polyacrylamide gel (4%C, 1.6%T), micro capillaries with 9.5% urea, 2% triton X-100 and ampholytes the same as mentioned earlier. The run conditions were strictly as per manufacturers instructions. On completion of the run, the 1st dimension gels were expunged from the glass capillaries and incubated for 15 min in SDS-PAGE sample buffer. The second dimension was run in Mini Protean III electrophoresis module (Bio-Rad). The profiles were monitored by staining the gels with coomassie brilliant blue and/or silver staining, as well as in ligand blot analysis.

Carbohydrate specific staining of receptors

The proteins extracted from the total BBMV of *Nephotettix sp.* by dissolving in 1% sodium deoxycholate were stained specifically for covalently bound oligosaccharides according to Moller [14]. This method involves periodic acid oxidation of the fixed proteins in SDS polyacrylamide gels, staining with Alcian Blue, and subsequent silver enhancement staining at high temperatures to specifically stain the oligosaccharides. Staining of non-glycosylated proteins was optional in the method and was not included for this experiment. Instead, coomassie blue staining was used to stain the total proteins, including the non-glycosylated proteins.

Ligand Blot Assay of deglycosylated receptor protein

Total BBMV proteins of *Nephotettix sp.* were subjected to deglycosylation [15], using the N-Glycosidase F deglycosylation kit (Roche) according to the protocol described in the kit manual. Each of total BBMV proteins (5 μg) was taken in a 0.5 ml microfuge tube, 10 μl denaturation solution (supplied) added to it, and the mixture was incubated in a boiling water bath for 3 min. The solutions were brought back to room temperature, reaction buffer (supplied) added to it, and the mixture was incubated at room temperature for 15 min. Recombinant PNGaseF (supplied) was added to the mix and incubated at 37°C for two hours. The deglycosylated samples were boiled with SDS-PAGE sample buffer and run in a 12% SDS-polyacrylamide gel. Non-deglycosylated BBMV proteins were loaded onto a separate well of the same gel as the positive control. Ligand blot analysis of the two samples was carried out as described before.

Results

Purification & SDS-PAGE analysis

All the lectins purified through affinity chromatography and ion-exchange chromatography were subjected to 15% SDS-PAGE analysis which shows single bands of ∼12 kDa. Western blot analysis performed by using anti garlic leaf lectin (ASAL) antibody also verified the purified lectins and suggested their cross-reactivity to anti-ASAL antibody.

Toxicity determination of lectins

From the *A. craccivora* bioassay experiments, it was evident that ASAL is comparatively more potent as a toxin than CEA and DEA (Figure 1A).The LC_{50} values of ASAL, CEA and DEA were 0.150, 0.184 and 0.212 nanomoles respectively. Bioassay experiments with *Nephotettix sp.* against ASAL also showed similar detrimental effects on the survival of the insect (Figure 1B).

Immunolocalization analysis

The immunolocalization results were monitored in an Olympus Compound Microscope with a camera mount at 40× magnification. Results were identical for sets of B and C, due to which, set C results are not shown here.

From Figure 2 it is evident that ASAL localizes itself in the epithelial membrane of the insect midgut in set B. The control sections of set A do not show any such attribute and validates the localization.

Ligand blot assay

BBMV proteins isolated from *Nephotettix sp.* and *A. craccivora* when analysed through ligand blot experiment with anti-ASAL antibody, receptor protein of ∼66 kDa, lighted up as demonstrated in Figure 3A. Ligand blot analysis of the respective BBMV proteins in two-dimensional PAGE yielded the same profile as in SDS-PAGE with additional information on the pI of the receptor proteins, which were found to be ∼6.5 (Figure 3B) and ∼8.5 (data not shown) respectively.

Carbohydrate specific staining

Figure 4A depicts the carbohydrate specific staining of BBMV protein of *Nephotettix sp.* Lane 1 shows specifically stained 66 kDa band of BBMV protein and lane 2 shows its subsequent coomassie stained profile.

Deglycosylation and subsequent ligand blot analysis

The total BBMV protein fraction of *Nephotettix sp.* was subjected to ligand blot assay after deglycosylation reaction using anti ASAL antibody. Figure 4B documented the loss of ligand specificity of BBMV receptor to ASAL after deglycosylation.

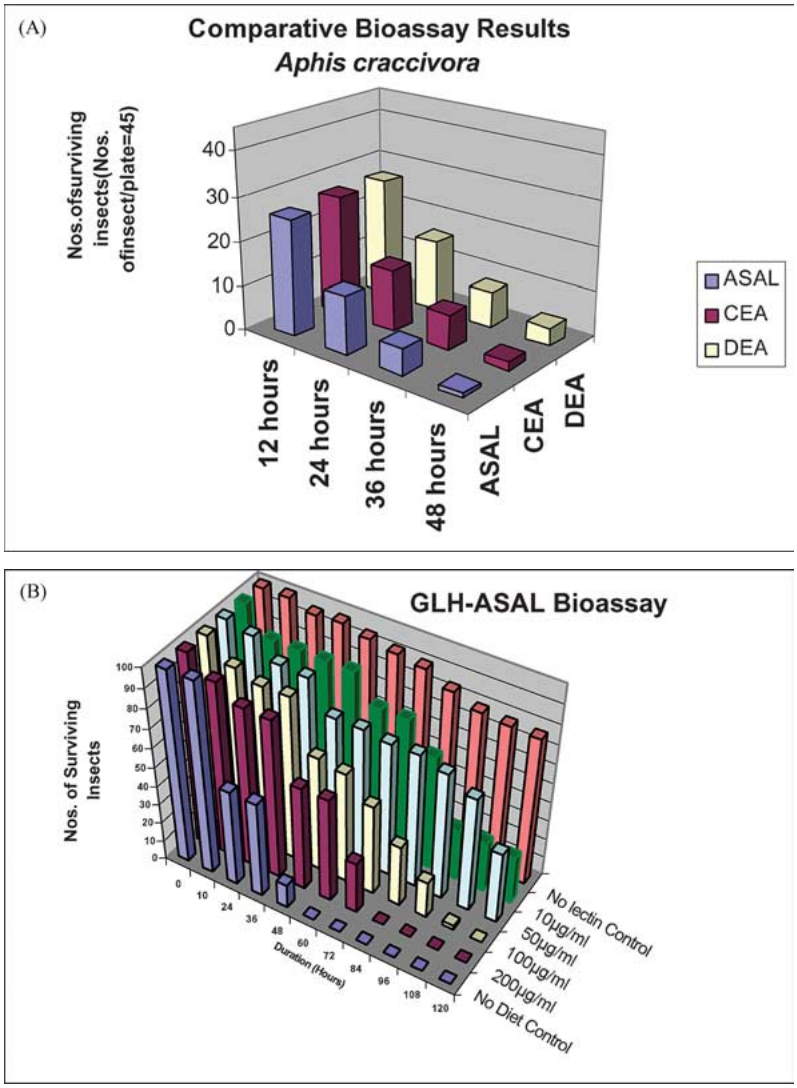

Figure 1. (A) Bioassay of *Aphis craccivora* with three lectins, ASAL, CEA and DEA in artificial diet. Bar showing the survival of insects at every twelve hours. (B) Bioassay of *Nephotettix sp*. (GLH) with different doses of ASAL. Bar showing the survival of GLH in diet supplemented with different conc. of ASAL.

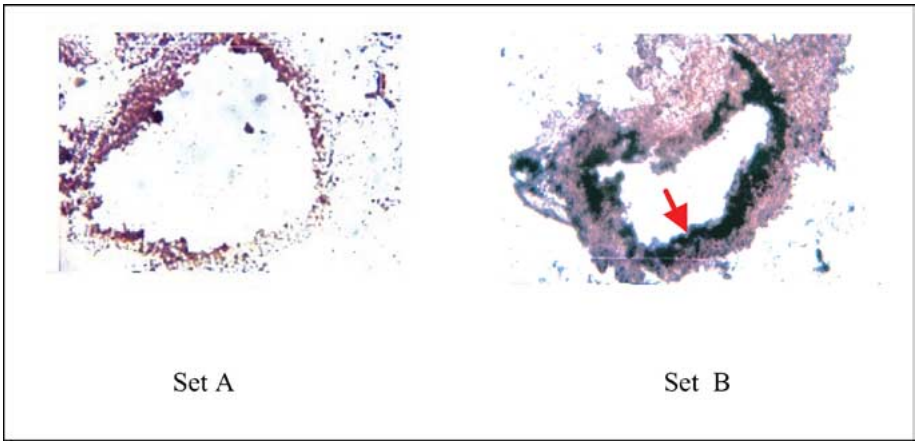

Figure 2. Immunolocalisation of ASAL at the luminial epithelium of *A. craccivora.* (A) Mid gut section of insect fed the artificial diet alone (B) Mid gut section of *A. craccivora* treated with 2 nanomolar ASAL. (red arrowhead showing the localization of ASAL in the luminial epithelium).

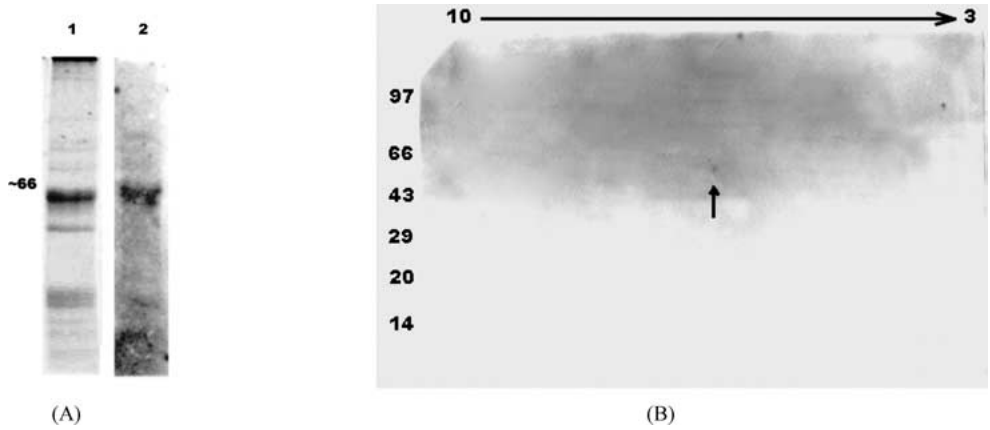

Figure 3. (A) Ligand Blot Analysis of the BBMV proteins. Panel 1: ~66 kDa receptor protein of GLH. Panel 2: ~66 kDa receptor protein of *A.craccivora*. (B) Ligand Blot Analysis of the receptor of GLH separated in a 2-dimensional-PAGE indicating a pI value of ~6.5.

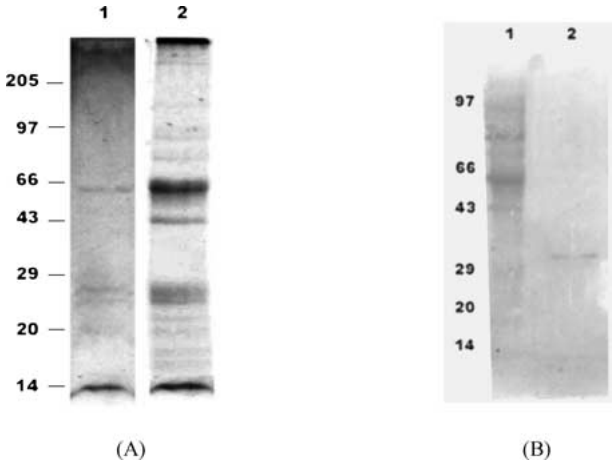

Figure 4. (A) Characterization of the GLH receptor as a glycoprotein. Lane 1: stained glycoproteins depicting ~66 kDa receptor. Lane 2: Coomassie stained total BBMV proteins. (B) Deglycosylation and subsequent ligand blot analysis of GLH BBMV proteins. Lane 1: ligand blot of BBMV proteins before deglycosylation. Lane 2: ligand blot shows no ASAL binding to ~66 kDa receptor, upon deglycosylation.

Discussion

Expression of the appropriate control agents in plants to protect them from target insects has been one of the objectives of the crop improvement program. Three severely damaging target homopteran insects *Nephotettix sp*. (Green Leaf Hopper of Rice), *L. erysimi* (Mustard aphid), *A. craccivora* (pea aphid) have not been well studied in terms of their susceptibility to so-far-reported control agents. This necessitated the identification and characterization of new control agents.

However, two mannose-binding plant lectins, *Galanthus nivalis* agglutinin (GNA) isolated from bulb of *Galanthus nivalis* (Amaryllidaceae family), and *Allium sativum* agglutinin (ASA) isolated from bulb of *Allium sativum* (Alleaceae family) have been earlier reported to have some activity against some insects, namely cowpea weevil (a coleopteran pest), rice brown plant hopper, and green leaf hopper [3–10]. With such background information, intensive efforts have been made to isolate a few more mannose-binding lectins from plant members of the two above-mentioned families to judge the efficacy of them against target pests as well as to monitor their mode of action on target insects. It has been apparent from our bioassay experiment that all three lectins are sufficiently effective to control the three target insects, albeit, comparative analysis of the LC_{50} values of three lectins suggest that ASAL is the most potent effective agent against all three target insects having the lowest LC_{50} value, which is followed by CEA and DEA. Our previous report has also identified the epithelial membrane of the midgut of the lectin treated red cotton bug as the primary binding site for ASAL for its affectivity [1]. The current study has been extended to immunolocalization analysis using anti-ASAL polyclonal antibody and similar binding feature of ASAL with midgut receptor protein has been experienced also in case of the present target insect, *Aphis craccivora*, the severely damaging pest of chickpea. Ligand blot analyses carried out with total BBMV of all three target insects using anti-ASAL polyclonal antibody, identified the receptor proteins of ASAL of approximate molecular weight of ~56 kDa [1], ~66 kDa, and ~66 kDa isolated from mustard aphid, green leaf hopper, and pea aphid respectively. The ligand positive receptor proteins were further resolved in 2–dimensional poly acrylamide gel and verified through ligand blot assay. The carbohydrate-specific staining of all three receptor proteins depicted through gel analysis established the fact that individual receptors are glycoproteins. Such receptors, when deglycosylated and further analyzed through a ligand blot experiment with anti-ASAL polyclonal antibody, failed to recognize and bind to mannose-specific ASAL, supporting their glycosylated characteristics. The binding affinity of ASAL to the insect BBMV receptor proteins, which facilitates efficient insect control ability, has been established using biochemical parameters. Thus, ASAL with its mannose-binding

ability provides itself as a potent candidate protein to be expressed in target plants to develop resistance against a range of homopteran pests.

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References

1 Bandyopadhyay S, Roy A, Das S, Binding of garlic (*Allium sativum*) leaf lectin to the gut receptors of homopteran pests is correlated to its insecticidal activity, *Plant Science* **161**(5), 1025–33 (2001).

2 Roy A, Banerjee S, Majumder P, Das S, Evaluation of the efficiency of different Mannose-binding plant lectins in controlling homopteran insect, Red Cotton Bug, *J Agric Food Chem* **50**, 6775–9 (2002).

3 Powell KS, Gatehouse AMR, Hilder VA, Gatehouse JA, Antimetabolic effects of plant lectins and plant and fungal enzymes on the nymphal stages of two important rice pests, *Nilparbarvata lugens* and *Nephotettix cinciteps*, *Entomol Exp Appl* **66**, 119–26 (1993).

4 Powell KS, Gatehouse AMR, Hilder VA, Gatehouse JA, Antifeedant effects of plant lectins and plant and an enzyme on the adult stages of the rice brown plant hopper, *Nilparbarvata lugens*, *Entomol Exp Appl* **75**, 119–26 (1995).

5 Hilder VA, Powell KS, Gatehouse AMR, Gatehouse JA, Gatehouse LN, Shi Y, Hamilton WDO, Merryweather A, Newell CA. Timans JC, Expression of snowdrop lectin in transgenic tobacco plants resulted in added protection against aphids. *Transgen Res* **4**, 18–25 (1995).

6 Gatehouse AMR, Powell KS, Van Damme EJM, Peumans WJ, Gatehouse JA, Insecticidal properties of plant lectins: Their potential in plant protection. In *Lectins. Biomedical Perspective*, edited by Pustzai AJ (Taylor and Francis, Basingstoke, UK, 1995).

7 Gatehouse AMR, Down PE, Powell KS, Sauvion N, Rahbe Y, Newell CA, Merryweather A, Hamilton WDO, Gatehouse JA, Transgenic potato plants with resistance to the peach potato aphid *Myzus persicae, Entomol Expl Appl* **79**, 295–307 (1996).

8 Goldstein IJ, Poretz RD, Isolation, physiochemical characterization and carbohydrate–binding specificity of lectins. In *The Lectins: Properties, Functions and Applications in Biology and Medicine*, edited by Liener IE, Sharon N, Goldstein IJ (Academic Press, New York, 1986), pp. 33–247.

9 Czapla TH, Plant lectins as insect control elements. Advances in insect control: The role of transgenic plants, edited by Carozzi N, Koziel M (Taylor and Francis, 1997).

10 Rao KV, Rathore KS, Hodges TK, Fu X, Stoger E, Sudhakar D, Williams S, Christou P, Bharathi M, Bown DP, Powell KS, Spence J, Gatehouse JA, Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown plant hopper, *The Plant J* **15**, 469–77 (1998).

11 Laemmli UK, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4, *Nature* **227**, 680–5 (1970).

12 Dadd RH, Mittler TE, Permanent culture of an aphid on a totally synthetic diet, *Experientia* **22**, 832 (1976).

13 Finney DJ, *Probit Analysis*, 3rd edition, Cambridge University press, Cambridge (1971).

14 Moller HJ, Poulsen JH, Staining of Glycoproteins/ Proteoglycans in SDS-Gels. In *Protein Protocols Handbook*, edited by Walker JM (Humana Press, New Jersey, 1996), pp. 627–32.

15 Banerjee S, Hess D, Majumder P, Roy D, Das S, The interaction of *Allium sativum* leaf agglutinin with a chaperonin group of unique receptor protein isolated from a bacterial endosymbiont of the mustard aphid, *JBC* (in press).