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Expression of snowdrop lectin in transgenic tobacco plants results in added protection against aphids

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The range of sap-sucking insect pests to which GNA, (the mannose specific lectin from snowdrops (*Galanthus nivalis*)) has been shown to be insecticidal in artificial diets has been extended to include the peach potato aphid (*Myzus persicae*). A gene construct for constitutive expression of GNA from the CaMV35S gene promoter has been introduced into tobacco plants. A transgenic tobacco line which expresses high levels of GNA has been shown to have enhanced resistance to *M. persicae* in leaf disc and whole plant bioassays, demonstrating the potential for extending transgenic plant technology to the control of sap-sucking insect pests.

Keywords: transgenic crops; insect resistance; aphid; homoptera; lectin; crop protection; *Nicotiana tabacum*

Introduction

Transgenic crops expressing foreign genes which confer added protection against insect pests could make a significant contribution within integrated pest management systems to provide sustainable agriculture for the future. Considerable progress has been achieved with transgenic crop plants which express *Bacillus thuringiensis* endotoxins (Barton *et al.*, 1987; Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987; Peferoen, 1992; Barton and Miller, 1993) and which express plant-derived proteins such as protease inhibitors (Hilder *et al.*, 1987; Johnson *et al.*, 1989; Hoffman *et al.*, 1992; Hilder *et al.*, 1993) or lectins (Boulter *et al.*, 1990) against insects which feed by chewing plant tissues. There are no reports so far, however, of the use of this technology to control sap-sucking insects belonging to the order Homoptera, which include some of the most devastating insect pests worldwide. These insects have piercing and sucking mouth-parts and feed upon sap. They include the aphids (Aphididae), whiteflies (Aleyrodidae), planthoppers (Delphacidae) and leafhoppers (Cicadellidae). Many species are serious pests of agricultural and horticultural crops and of ornamental plants. Crop damage is caused not only as a direct result of feeding, but also the injury caused by insertion of the mouthparts produces lesions through which a variety of plant pathogens may enter. Some of the most serious damage caused by these pests is due to their role as vectors of plant viruses.

Two conditions must be satisfied in order to produce transgenic plants with enhanced resistance to sap-sucking insects. First, gene products must be identified which are effective against sap-suckers and whose encoding genes can be obtained and transferred. Secondly, an effective means of delivering the chosen gene products to the insects, which feed exclusively on phloem sap, must be developed for use in transgenic plants.

We have recently demonstrated that GNA – the lectin from snowdrop (*Galanthus nivalis*) – is insecticidal to representatives of two quite distinct families of sap-sucking insect pests of rice, the rice brown planthopper (*Nilaparvata lugens*) and the rice green leafhopper (*Nephotettix cinciteps*) (Hilder *et al.*, 1992; Powell *et al.*, 1993). We here report on the effectiveness of GNA

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against a member of yet another distinct family of homopteran – the family Aphididae – represented by the peach potato aphid (*Myzus persicae*), both in an artificial diet bioassays and in transgenic tobacco plants expressing an introduced GNA gene under the control of the cauliflower mosaic virus 35S gene promoter. The Aphididae are the most important group of plant bugs and are better adapted for life in the temperate zones than the tropics. *M. persicae*, the peach potato or green peach aphid, is a serious, polyphagous pest worldwide, and acts as the vector for over 100 different disease-causing viruses. Many biotypes show multiple resistance to the conventional chemical pesticides (see Hill, 1987). The demonstration of increased resistance of transgenic plants to this aphid provides the potential for expanding transgenic crop technology to the control of sap-sucking insect pests.

Materials and methods

Insects

The source of the laboratory stocks of *M. persicae* was an adventitious infestation of tobacco plants in one of the plant growth rooms at Durham University. Aphids were maintained on mature plants of *N. tabacum* cv. Samsun NN in a controlled environment growth chamber, 24 ± 2° C, 16 h day, 75% relative humidity (RH).

Plants

Control tobacco plants were produced as clonal replicates by stem cutting from a seed-derived plant of *N. tabacum* cv. Samsun NN. Transgenic plants were derived from the regenerated tobacco plant resulting from a single transformation event by stem cutting from transformed *N. tabacum* Samsun NN-15GNA35, described below. Insertion of the GNA-expression construct was at more than one locus in this line ($P > 0.05$ for the null hypothesis of 2 Mendelian loci) as determined by segregation analysis of kanamycin resistance in seeds from selfed 15GNA35.

Plants were maintained in 5-inch pots of loam-based compost in controlled environment growth chambers as described for insect maintenance.

Lectin assays

Leaf proteins were extracted from ground fresh or freeze-dried leaf tissue in 50 mM Tris-HCl, pH 9.2. Protein concentration of the samples was determined by the Bradford assay (1976).

GNA content of samples was determined by dot-blot immunoassay using polyclonal rabbit anti-GNA antiserum as the primary antibody and visualised by enhanced chemiluminescence (ECL) using HRP-conjugated goat anti-rabbit secondary antibody (Bio-Rad Laboratories Ltd., Watford, UK) and an ECL signal detection kit (Amersham International plc., Amersham, UK).

Haemagglutination assays for lectin functional activity were performed against trypsinised rabbit erythrocytes as described by Lis and Sharon (1972).

Artificial diet bioassays

The artificial diet used was that described by Mittler and Dadd (1962) for the rearing of aphids. Snowdrop lectin (Vector Laboratories, Peterborough, UK) was incorporated at 0.1% w/v. Controls containing no protein and others containing no diet were set up for each trial. The diet solution was incorporated into feeding vessels based on those described by Mitsuhashi (1974). Plastic Petri dishes, 35 mm diameter, were lined with water-soaked filter paper. Five neonate aphids were transferred from the host plant with a moistened camel hair brush and the vessel sealed with a stretched Parafilm™ feeding sachet containing 200 µl diet solution. The feeding sachet was put under hydrostatic pressure by incorporating a 5 cm head of water over the sachet. Diets were changed daily to ensure a fresh nutrient supply. Feeding chambers were maintained in the constant environment growth chamber and the number of surviving nymphs was recorded daily. Ten replicates of each treatment were performed in each of three trials. Aphids on the control diets survive for longer than 14 d and newly laid nymphs appear on these diets after 8 d.

The increase in mortality, corrected according to Abbott (1925) on the day when all the 'no-diet' aphids had died was used as the measure of effectiveness of the test protein, as described in Powell *et al.* (1993).

Leaf disc bioassays

Clonal replicates of transgenic tobacco line 15GNA35 and untransformed tobacco controls were produced from stem cuttings and grown in 5-inch pots in the controlled environment growth room. When the plants were ca. 40 cm tall, discs of 25 mm diameter were cut from fully mature leaves, avoiding areas of leaf with large veins. Leaf discs were floated upside-down on 10 ml tap water in 5 cm diameter disposable Petri dishes. Two late instar, alate aphid nymphs were transferred to each leaf disc with a camel hair brush, the dishes were lidded and maintained in the controlled environment growth chamber. Aphids were counted daily.

Whole plant bioassays

Clonal replicates of transgenic tobacco line 15GNA35 and untransformed tobacco controls were produced from stem cuttings and grown in 5-inch pots in individual 24 × 24 × 40 cm perspex boxes within the controlled environment growth room. When the plants were ca. 20 cm tall they were infested with 8 late instar, alate aphids. Growth of the aphid populations was monitored by counting all aphids on each plant. In the experiments described, 6 control and 6 transgenic plants were used.

Statistical analyses

Association was tested by a G-test. Difference between means of normally distributed data were tested by type 1 ANOVA. The coefficient of variation (CV) was used to compare the variability of different populations. Non-parametric tests were not required in the reported studies.

Results

Artificial diet studies

The insecticidal effect of GNA against peach potato aphids has been tested at 0.1% w/v in a wholly defined artificial diet under hydrostatic pressure. This level of GNA substantially reduces survival of neonate peach potato aphid nymphs on these artificial diets (Fig. 1A). The corrected mortality, measured as defined in the Materials and methods section on the day when all starved aphids had died, was 33 ± 1%. This is less than that which has been measured for GNA against the brown planthopper (79%) or green leafhopper (87%) (Powell *et al.*, 1993), but is nevertheless significant ($G_{adj} = 13.662$: $p_{[H0:\ EXP\ =\ CON]} < 0.001$).

GNA also has deleterious effects on the development of those insects which do survive. The mean size of surviving nymphs was significantly lower than those fed on control diet (Fig. 1B). Such a reduction in size will have serious effects on the development time and subsequent fecundity of these insects and hence on the rate of aphid population growth.

GNA-expressing transgenic tobacco plants

Recombinant cDNAs encoding the precursors of several isoforms of GNA have been cloned (Van Damme *et al.*, 1991). The (near) full length clone pLECGNA2, encoding the GNA isolectin GNA2 was selected for transfer to transgenic plants. A fragment corresponding to the entire coding sequence, including the 23 residue amino- and 22 residue carboxy-terminal extensions which are cleaved from the mature lectin was cloned behind the CaMV35S gene promoter in the binary vector pRok2 (Baulcombe *et al.*, 1986) (Fig. 2A). This construct was introduced into tobacco by standard *Agrobacterium*-mediated leaf disc transformation (Horsch *et al.*, 1985). Regenerated tobacco plants of the transformed line 15GNA35 were identified as expressing high levels of the snowdrop protein, in initial immunoassays of leaf protein extracts. These extracts had a high haemagglutinating activity against trypsinized rabbit erythrocytes, demonstrating the functional integrity of the lectin. A more detailed investigation of the pattern of GNA expression in clonal replicate plants from this line revealed that the measured level of GNA varied enormously with leaf position on the stem (Fig. 2B), being much higher in older, mature leaves than towards the growing tip of the plant. Note that this 'within plant' variability (i.e. between

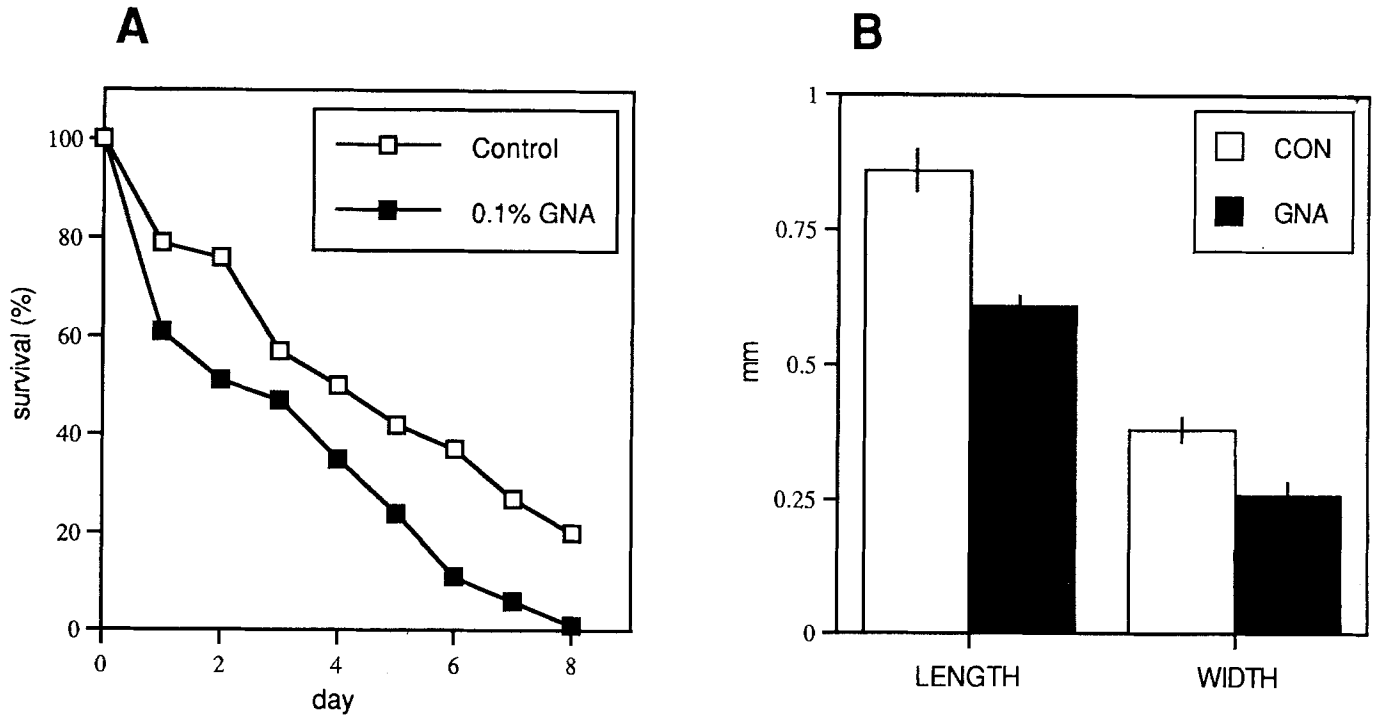

Fig. 1. Artificial diet bioassay of snowdrop lectin against *M. persicae*. A: Insect survival. B: Mean size (mm ± SEM) of surviving insects after 4 days. Dimensions of living insects were measured by image analysis after 4 days on the diet.

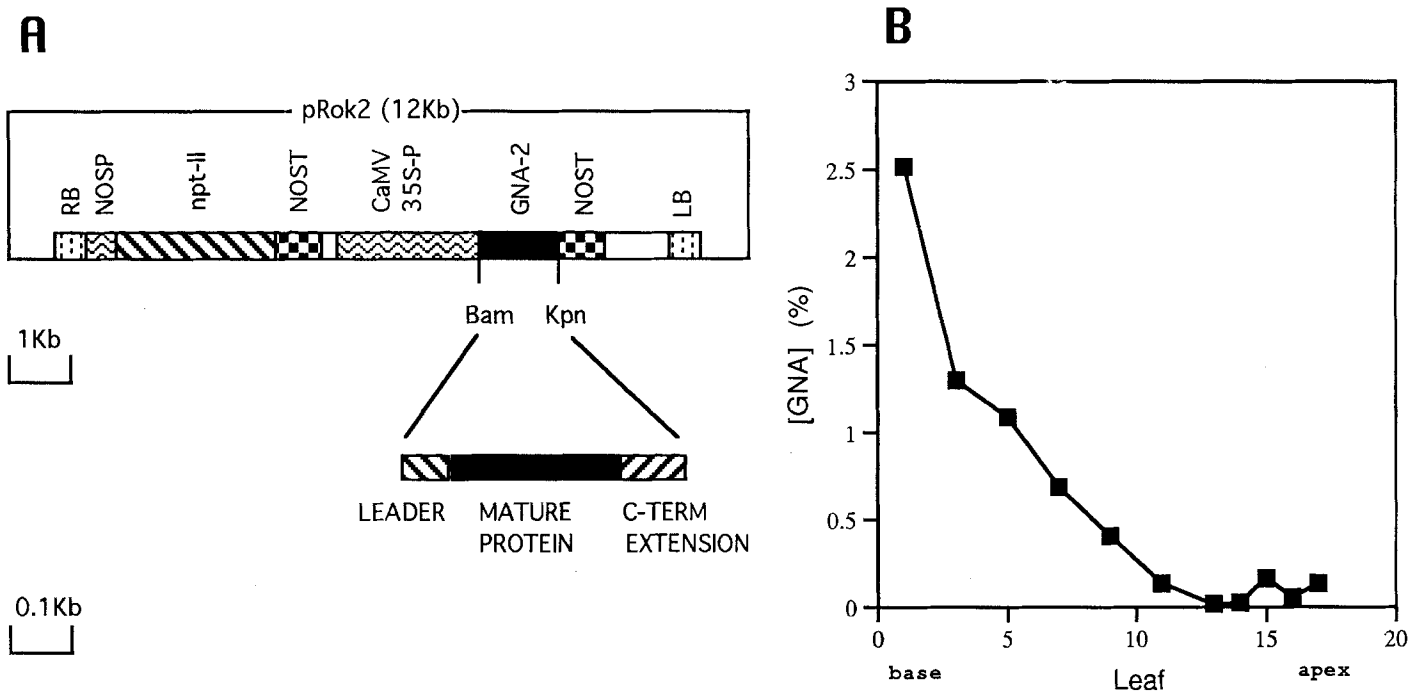

Fig. 2. GNA expression in transgenic tobacco. (A) Gene map of the T-DNA region in plasmid pRok9GNA1, for constitutive expression of GNA in transgenic plants. Elements illustrated are: RB – Ti plasmid right border repeat; NosP – nos gene promoter; Nos-T – nos gene terminator; CaMV35S-P – CaMV 35S gene promoter; GNA-2 – pro-lectin coding sequence from pLECGNA2; LB – Ti plasmid left border repeat. (B) Concentration of GNA (%w/w leaf total soluble protein) in different leaves of transgenic tobacco plants.

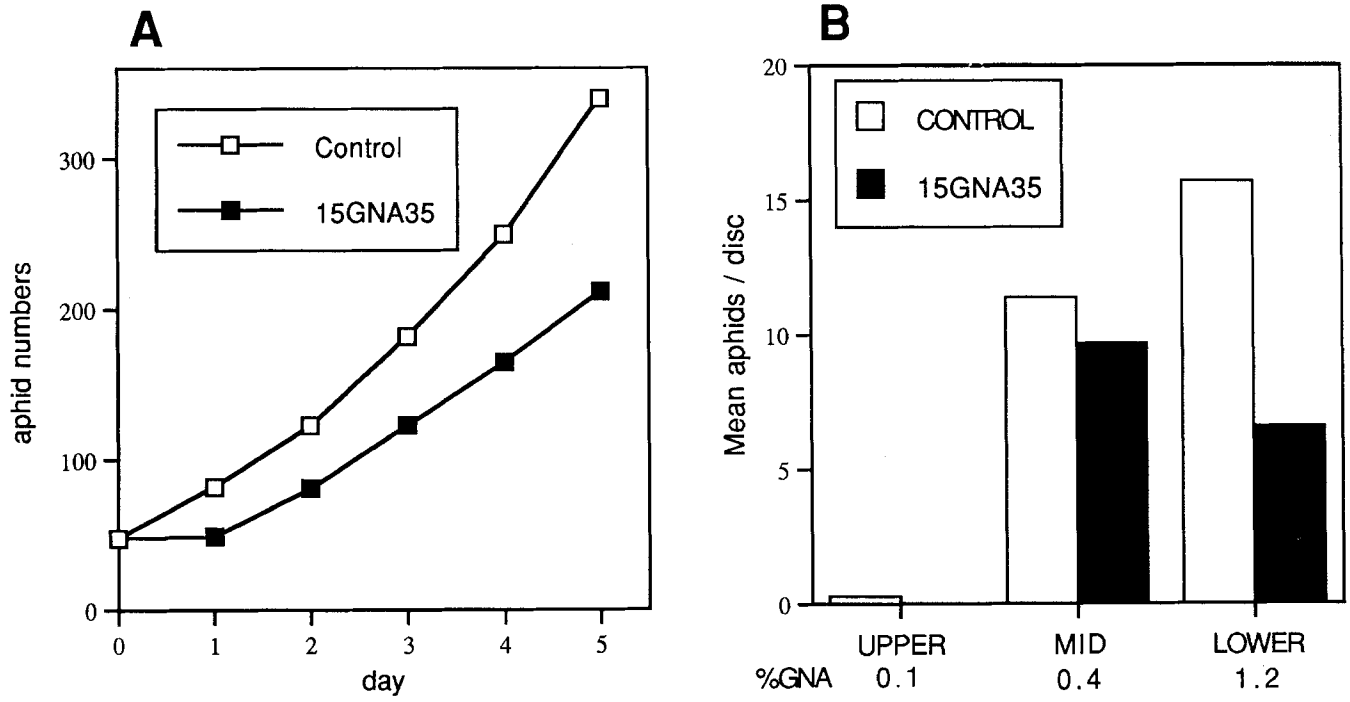

Fig. 3. Transgenic tobacco plant leaf disc bioassays against *M. persicae.* A: Aphid population growth in leaf disc bioassays with mature leaves; B: Aphid population growth in relation to leaf position.

different leaves from the same plant: coefficient of variation (CV) = 130.0% is much greater than the variation from 'within leaf' (i.e. between different samples from the same leaf: CV = 10.3%) or from 'within population' (i.e. between the equivalent leaf from different replicate plants: CV = 8.9%).

The original purpose for making these plants was to test the efficacy of constitutive expression of GNA in transgenics against leaf eating lepidopteran pests. However, immunocytological investigation of leaf and petiole sections has shown that this line had a high level of expression of the lectin in vascular tissue, including the phloem, relative to the mesophyl, and GNA has been detected in the honeydew produced by aphids feeding on the GNA-expressing plants (Shi *et al.*, 1994). It was, therefore, considered worthwhile testing them against a phloem-feeding pest.

Insect bioassays on transgenic plants

Leaf discs were cut from large, mature leaves of control and transgenic plants and used to carry out bioassays with late instar aphid nymphs. Mature leaves were selected as a preliminary study has shown these to be the preferred feeding site for aphids on (non-flowering) control tobacco plants. A significant reduction in the size of the aphid population on the transgenic leaf discs was evident (Fig. 3A), and the rate of growth of the aphid population, calculated as the doubling time (T^D), was slower on leaf discs from transgenic plants (T^D = 2.47 days) than on the controls (T^D = 1.62 days).

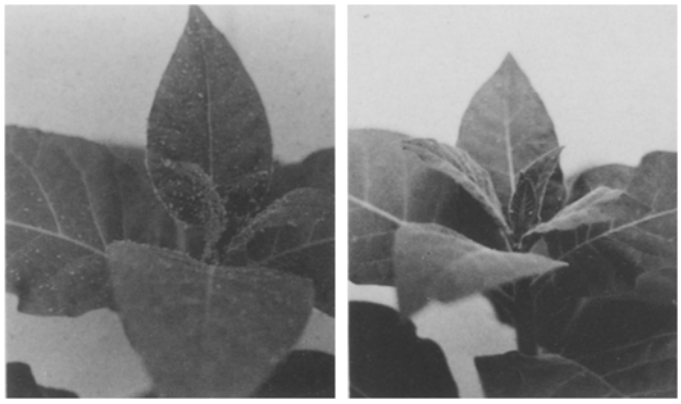

Fig. 5. Transgenic tobacco whole plant bioassays against *M. persicae.* Apices of untransformed control plant (left) and GNA-expressing transgenic (right) 34 days after infestation.

Further leaf disc bioassays were performed to establish the extent of variation in protection afforded between different replicate plants. The mean number of aphids per leaf disc after 7 days bioassay was again significantly lower on discs taken from mature leaves of transgenic plants (8.3 ± 2.5) compared to controls (38.8 ± 8.6). The coefficient of variation in aphid numbers per disc for different replicate plants was 54%. A further bioassay was performed wherein the discs were taken from leaves at different parts of the plants (Fig. 3B). The aphids performed much better on mature leaves taken from

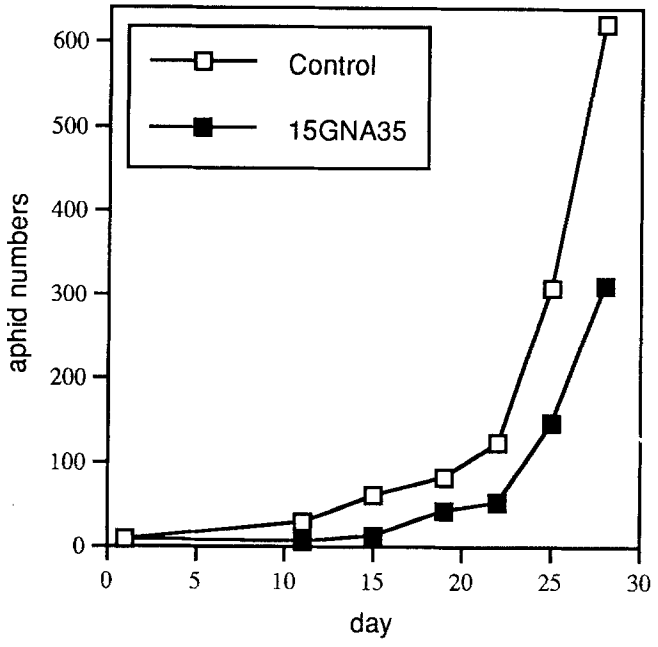

Fig. 4. Transgenic tobacco whole plant bioassays against *M. persicae.* Aphid population growth on control and GNA-expressing transgenic plants.

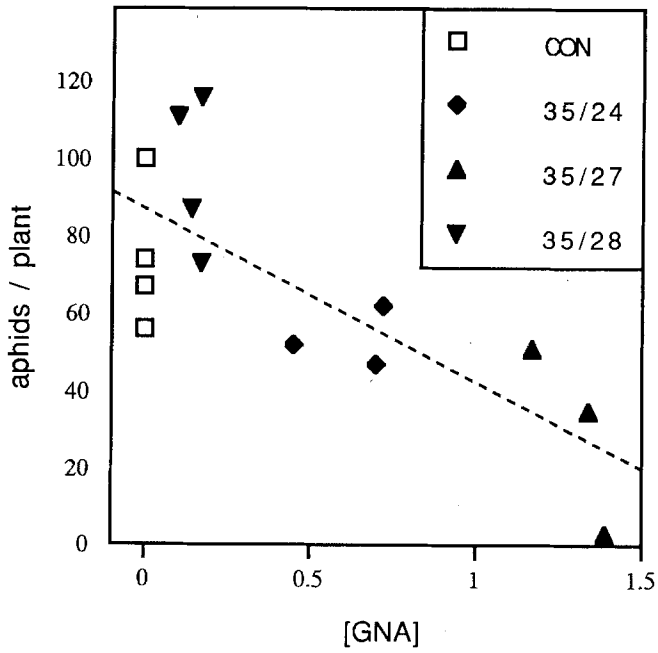

Fig. 6. Effect of GNA expression level in segregating progeny of transgenic tobacco on aphid population growth in whole plant bioassay.

Table 1. Leaf disc bioassay of control and GNA-expressing transgenic tobacco plants against *M. persicae* conditioned on control or GNA-expressing transgenic tobacco plants

Source of leaf disc	Mean aphids / disc [± SEM] Source of aphids	
	Control	15GNA35
Control	11.8 ± 3.0	12.0 ± 2.8
15GNA35	3.3 ± 1.8	2.5 ± 1.8

control plants than on young leaves, reflecting their preferred distribution on control tobacco plants. The 'effectiveness' of the transgenics in reducing aphid population build-up was much more marked in the lower, older leaves, reflecting the pattern of GNA concentration described above.

When whole tobacco plants were infested with 8 late instar nymphs and maintained in sealed individual plantaria, the growth of the aphid population on the transgenic plants was significantly reduced (Fig. 4), mirroring the results obtained in leaf disc bioassays. The rate of population growth on the transgenic plants was slowed to $T^D = 4.8 \pm 0.2$ days compared with 4.4 ± 0.2 days on the controls. The difference which this made to apparent greenfly infestation may be judged from Fig. 5.

Self set seed from plants of line 15GNA35 was planted and 20 progeny screened immunologically for GNA levels. Three plants, identified as high, intermediate and low/non-expressers, were clonally propagated (along with untransformed controls to provide replicates for a further whole plant bioassay. After 21 days the GNA content of a mature fully expanded leaf from each plant was measured. The inverse correlation between aphid population growth and GNA content is illustrated in Fig. 6 ($r = 0.760$; $p_{[H0: r = 0]} < 0.01$).

It is clearly important to establish whether those aphids which do survive on the GNA-expressing transgenic plants represent an inherently resistant subgroup (despite the expectation that the aphid test population was essentially clonal). Therefore, leaf disc bioassays were carried out to compare the performance of aphids which had been maintained on control tobacco plants with those which had been maintained on GNA-expressing transgenic tobacco plants for a period of six weeks, during which time the aphids had gone through several generations. The results (Table 1) show that 'GNA-conditioned' aphids are equally susceptible to the effects of GNA expression in transgenic tobacco plants.

Discussion

GNA at 0.1% has now been shown to be antimetabolic in artificial diets to members of three distinct families of homopteran pests. This concentration of the protein was selected as the base-line level for testing because, although the concentration of proteins in phloem sap is generally very low, in some cases proteins are present in sap at (at least) this level (e.g. Read and Northcote, 1983) and it should, therefore, be possible to obtain promoters which direct expression of foreign genes in the sap of transgenic plants at this level.

Lines of transgenic tobacco have been produced which express GNA at high levels – up to ca. 2.5% of leaf total soluble protein. We are not, as yet, able to translate this into a measure of concentration in the phloem, but immunocytological studies indicate it to be high. The pattern of expression of the foreign gene in this construct was unexpected by comparison with a similar construct containing the cowpea trypsin inhibitor (CpTI) gene, where CpTI expression was measured highest in young leaves but where the overall variation between leaves within a plant was considerably less than observed here (Hilder *et al.*, 1990). This does, however, appear to be the general pattern of expression obtained with this construct in tobacco, as it has recently been demonstrated, though at a lower overall level, in the single GNA gene insertion line 15GNA56 (data not shown). It should be noted that by 'expression' we are more strictly measuring accumulation of GNA, which will be determined not only by promoter activity but also by stability and transport of the protein.

Such a marked pattern of variability in expression has serious consequences for the effectiveness of the transgenics in relation to the feeding preference of any target pest. These plants would be of little use in the control of any pest which fed exclusively on the growing tip, however susceptible it might be to GNA. This highlights the importance of an appreciation of the wider biology of crop/pest interactions if transgenic crops are to be seriously deployed.

The bioassays reported here demonstrate that proteins which have been identified as being effective against homopterans in artificial diets are also effective against these insects when expressed in the phloem of transgenic plants. This opens up the area of the control of this important group of agricultural pests, which alone account for some 26% of the world-wide insecticide expenditure, to the transgenic crop strategy. We consider that the transgenic plants reported here do not represent an optimal application of this technology to sap-sucking insects – constitutive expression of the protein from the CaMV35S gene promoter was employed with a view to controlling leaf-chewing insects. We are currently producing and testing plants which express aphicidal proteins from phloem specific promoters, such as the rice sucrose synthase 1 gene promoter (Wang *et al.*, 1993), as a next step towards genetically engineering insect resistance for the control of this important order of pests.

Preliminary results similar to those reported here have

been obtained from transgenic potatoes and lettuces transformed with pRok9GNA1. A more detailed examination of the mechanism of enhanced aphid resistance in GNA-expressing transgenics is being carried out in these crop species.

It would be naive to suppose that the level of protection reported here which is afforded to these plants in the laboratory by the constitutive expression of the snowdrop lectin would be sufficient to provide commercially acceptable protection of crops from sap-sucking insects. What an acceptable level of protection actually is can only be assessed by extensive, meaningful testing in the field. However, as one component within an integrated pest management system, even the level of reduction in rate of build up of the pest population observed here would permit greater flexibility in the employment of other control measures and perhaps contribute to a more sustainable agricultural system.

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