Genetic engineering of rice for resistance to homopteran insect pests

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The rice brown planthopper (BPH; *Nilaparvata lugens*) is a serious pest of rice crops throughout Asia, damaging plants both through its feeding behavior and by acting as a virus vector. Like many homopteran pests of crops, it is primarily a phloem feeder, abstracting sap via specially adapted mouthparts. An artificial diet bioassay system for this pest was developed to allow the effects of potentially insecticidal proteins to be assayed. Several lectins and oxidative enzymes were found to be toxic to BPH. Snowdrop (*Galanthus nivalis*) lectin (GNA) was selected for further study as it is nontoxic to higher animals. A cDNA encoding GNA was assembled into constructs for expression in transgenic plants, with the aim of producing transgenic rice plants that would express the foreign protein in their phloem sap and be resistant to BPH. Constitutive expression of GNA in model plant systems was shown to have deleterious effects on the development of lepidopteran and homopteran pest insects. Phloem-specific promoters for expressing GNA in transgenic rice were isolated and characterized with the aim of increasing the effectiveness and specificity of the protection against BPH. A construct containing the GNA coding sequence driven by the promoter from the rice sucrose synthase *RSs1* gene was tested in tobacco and transformed into rice. Transgenic rice plants containing this construct are currently being evaluated.

Sucking insects of the order Homoptera can cause serious damage to rice, both directly and by acting as vectors for plant pathogens. The major pests in this order are the rice brown planthopper (BPH, *Nilaparvata lugens*) and the rice green leafhopper (GLH, *Nephotettix* spp.). Both BPH and GLH are economically serious pests of rice and can be the major cause of crop losses. Control by chemical insecticides is incomplete and, in any case, too expensive for poor farmers. It also poses health and environmental risks. Biological control and especially breeding for resistance are attrac-

tive alternatives to chemicals, but both methods could be augmented by genetic engineering. Insects can rapidly adapt to become resistant to control measures, so it is essential to use a pluralistic approach. It is now widely recognized that genetic engineering of "exotic" resistance genes is a significant new approach that offers possible solutions within several years.

Most work on resistance of plants to sucking pests has concentrated on the role of semiochemicals and plant secondary metabolites as feeding deterrents. The feasibility of engineering transgenic plants to confer the ability to produce secondary metabolites has yet to be demonstrated, and the ability to do this on a routine basis for given secondary compounds is in the future due to the complexity and species-specificity of the biochemical pathways involved—although this approach is now being addressed (Hallahan et al 1992). For some insect pests, the expression of *Bacillus thuringiensis* (*Bt*) endotoxin genes in transgenic plants has been shown to be an effective means of control, although the long-term use of *Bt* may depend on devising suitable management strategies to delay the buildup of *Bt*-resistant insect populations. However, sucking insects are not amenable to control by *Bt* bacteria, or toxins, at present, since no reported strain of *Bt* is effective against homopterans. To tackle the problem of producing transgenic plants with resistance to sucking pests, it was necessary to go back to insect bioassays. Products of genes that could be obtained reasonably easily, and which could be expressed in transgenic plants using existing technology, were assayed for their effect in artificial diet bioassay.

We have shown that certain plant lectins and enzymes have insecticidal properties toward BPH and other homopteran insect pests, and have assembled the necessary elements of a technology for producing transgenic rice plants with engineered insect resistance. The main emphasis in the program has been the production of rice with resistance to BPH, as this was identified as the pest most difficult to control using insecticides, to which it readily developed resistance. Although the use of biological control measures and the breeding of new resistant rice varieties have prevented BPH from becoming an uncontrollable pest, it remains a serious problem to rice growers throughout Asia.

Identification of insecticidal proteins

Insect bioassays

BPH feeds exclusively on the phloem and xylem saps of rice plants, with the phloem sap only providing a source of nutrients. An artificial diet system for this insect must thus mimic its natural foodstuff. A liquid diet formulation, containing sucrose, amino acids, and vitamins is used; portions of this diet are enclosed in parafilm sachets (which can be put under pressure, to simulate the normal phloem pressure in the plant), and the insects feed by probing the parafilm and sucking the diet in the same way in which they normally probe plant tissues and suck phloem sap. The diet allows the insects to develop through several nymphal stages to adults quite successfully with survivals of more than 50%, but is not suitable for rearing successive generations of insects (Powell et al 1993).

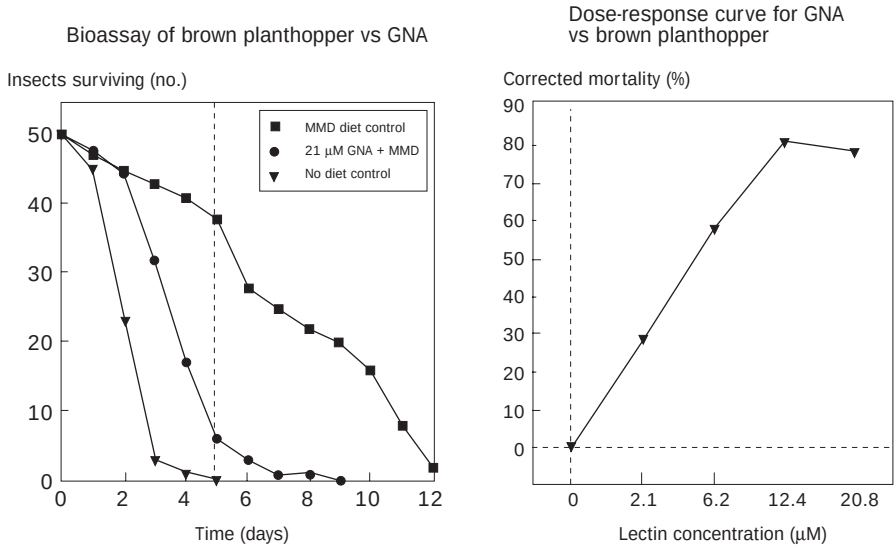

Fig. 1. Bioassay of BPH on artificial diets containing snowdrop lectin (GNA).

Bioassays of potentially insecticidal proteins are done by incorporation into the liquid diet, and the survival of insects is compared on control diet, diet + treatment, and a "no diet" control where the insects are given moisture but no nutrients. The "no diet" control allows the corrected mortality for the treatment to be calculated (see Fig. 1).

At the time when mortality on "no diet" = 100%, corrected mortality %/100 = 1 - {([survival on control] - [survival on treatment])/ survival on control}.

Standard statistical methods can be used to evaluate the significance of corrected mortality figures; however, these techniques are relatively insensitive and analysis of the survival vs time curves for treatments and controls can be used to identify more subtle effects on insect development. Some typical results for the insect bioassay are shown in Figure 1, which demonstrates that an effective insecticidal protein can be expected to give corrected mortality figures in excess of 50%.

Proteins with insecticidal properties toward BPH

Assay of a number of plant and other proteins against BPH in the bioassay system described above showed that the presence of an inert protein, such as ovalbumin, had no deleterious effects on survival, but some biologically active proteins were toxic (Powell et al 1993). Inhibitors of digestive enzymes, such as cowpea trypsin inhibitor and wheat α-amylase inhibitor, had no effect, as would have been expected on the basis that sap-sucking homopteran insects do not rely on protein or starch digestion for nutrients. On the other hand, two types of protein did show deleterious effects:

lectins and oxidative enzymes such as lipoxidase, and, to a lesser effect, polyphenol oxidase. The toxicity of lectins varied considerably from those that had very little effect on the corrected mortality at the concentration used (0.1% w/v in the liquid diet), e.g., the lectin from garden pea, to those that gave corrected mortality values of nearly 90%, e.g., the lectins from wheatgerm and from snowdrop.

The results of many similar bioassays have suggested that BPH is generally sensitive toward insecticidal proteins, so that results obtained with this species must be extended with caution to other insect pests. Nevertheless, assays with GLH showed that the lectins from snowdrop and wheatgerm were both strongly toxic toward this species also, although it was not sensitive to lipoxidase.

Toxicity of lectins

The bases for the toxicity of lectins toward animals, in general, are still the subject of research. In higher animals, binding of lectins to gut epithelial cells is well demonstrated, and effects on the growth of gut tissues, particularly in terms of effects on the normal structures of villi, are well documented (Pusztai 1991). Certain lectins also show systemic effects by crossing the gut wall intact and passing into the circulatory system. An additional factor is the effects of lectins on the attachment of gut microflora to the gut epithelium, which can lead to breakdown of the gut wall and bacterial invasion of gut tissues. All these effects are thought to be mediated through the carbohydrate-binding properties of lectins, which lead to interactions with cell surface glycoproteins, both on gut epithelial cells and on bacteria.

The situation in insects is less clear. Binding of lectins to gut surfaces in insects has been observed by several researchers, but the results of this binding are not characterized. The toxicity of wheatgerm lectin toward a range of insects and its specificity of binding toward chitin have led to suggestions that the peritrophic membrane, a thin porous chitin layer that covers the gut epithelium in many insects, is the target of its action. However, other chitin-binding lectins are not toxic and lectins with other carbohydrate-binding specificities are toxic (Powell et al 1993, 1995b).

The toxicity of many lectins toward higher animals limits their usefulness in the protection of crop plants that are intended for consumption. In particular, wheatgerm lectin, which is strongly insecticidal, is also significantly toxic to mammals and other higher animals. However, certain lectins, in particular those from the plant family Amaryllidaceae, show low or no toxicity toward higher animals, but are toxic to insects. This type of lectin is exemplified by snowdrop (*Galanthus nivalis*) lectin (GNA), which had been identified as toxic to BPH. GNA was thus selected as the "best candidate" gene for engineering of BPH-resistant rice.

Snowdrop (*Galanthus nivalis*) lectin (GNA)

Snowdrop lectin (like other Amaryllidaceae lectins) binds specifically to mannose residues in α-1,3 or α-1,6 glycosidic linkages. The protein is a tetramer of polypeptides of M_r approx. 11, 600, and is accumulated in snowdrop bulbs, and to a lesser extent in other tissues. It is encoded by a multigene family and many isomeric forms are present in snowdrop tissues. The polypeptides are synthesized as preproproteins and are sub-

ject to both cotranslational N-terminal processing and post-translational C-terminal processing. GNA protein and its encoding genes have been extensively characterized in the laboratory of W. Peumans and E. van Damme (Leuven, Belgium), from where a cDNA clone containing the complete coding sequence of a GNA isoform was obtained (van Damme et al 1991).

To generate a subclone containing only the GNA coding sequence, with convenient restriction sites for construct assembly, polymerase chain reaction (PCR) amplification of the desired sequence, with appropriate primers containing added restriction sites, was carried out. PCR products were cloned into pUC vectors, and selected clones were checked for PCR errors by DNA sequencing. The clone selected was identical in sequence to the reported GNA coding sequence, apart from one silent base change.

Effects of GNA on BPH

Further assays carried out with BPH were used to estimate the lower limit of effectiveness of GNA in an artificial diet. At 0.05% (w/v), the protein is as effective as at 0.1%, but below this level effectiveness declines with an LC_{50} of approximately 0.02%; this is equivalent to a protein concentration of approximately 6 mM (Fig. 1). Elevated levels of GNA do not increase insect mortality beyond approximately 90% in this system, although this reflects the way in which mortality is measured, rather than an ability of the insect to survive the treatment, since all insects on the GNA diets (at GNA concentrations of $\geq$0.05%) die within 7-10 d. Effects on insect survival are significant at the lowest levels tested (0.01% w/v; approx. 3 mM).

GNA has antifeedant properties toward BPH. This has been shown in two ways: first, indirectly, by measuring the production of liquid excreta, or honeydew, as an indication of food ingested (Powell et al 1995a); and secondly, directly, by examining the feeding behavior of the insect by the electrical penetration graph method. Honeydew production suggests that insects exposed to GNA at 0.1% (w/v) ingest virtually no diet over an initial 24-h period, and a reduced amount compared with control diet for the next 12 h. After this, ingestion approaches control levels. Similarly, the feeding behavior data also show a failure to ingest a diet containing GNA during a 4-h exposure period; whereas control insects spend approximately 25% of their time ingesting diet, of which approximately 70% is spent in actively sucking in the liquid diet, GNA-fed insects spend only 3%, and no active ingestion was observed. The decreased palatability of diet containing GNA seems unlikely to account for the high levels of mortality observed with this lectin; a more likely explanation is that once the insect is forced to feed on GNA-containing diet, a toxic effect then manifests itself. The shape of the survival curve with GNA-containing diet also suggests that an initial lag phase (when the insect is deterred from feeding) in the toxic effect of GNA is present.

An antifeedant effect might be viewed as disadvantageous in transgenic plants, since it will increase movement of hoppers and probing actions, and could thus increase the possibility of virus transmission. However, virus transmission by homopterans is not instantaneous; the time for transfer varies with virus, vector, and

host, but many viruses need prolonged feeding for efficient transfer to take place, which would be prevented by an antifeedant effect.

Expression of insecticidal proteins in transgenic plants

Phloem-specific promoters

Although the constitutive CaMV35S gene promoter, used in many constructs for expression in transgenic plants, is expressed efficiently in phloem tissue, it was felt desirable to identify promoters that would show phloem-specific expression for use in producing rice with BPH resistance. Use of such promoters could give higher levels of expression in the phloem and would minimize exposure of nontarget insects and other consumers of the plant material to GNA. Use of an endogenous phloem-specific promoter was decided on. Protein concentrations in phloem of different plant species have been estimated at 0.03-0.2% (w/v) in most species or as much as 10% in cucurbits, and thus the lower limits of effectiveness of GNA lie within achievable expression levels.

Sucrose synthase is known to be specific to phloem tissue and studies on the gene that encodes the enzyme from maize had suggested that the promoter was active and phloem-specific. A gene, designated *RSs1*, corresponding to the maize *Sh1* locus was isolated from rice, and fully characterized and sequenced (Wang et al 1992). The promoter sequence from this gene has been fused to the glucuronidase (*gus*) gene coding sequence in a promoter-reporter gene construct, and transformed into tobacco plants by standard techniques. Histochemical staining of the transformed plants with X-glc has shown that the *RSs1* promoter fragment used (approx. 1.2 kb of 5' flanking sequence, the transcription start, the first intron and the translation start) is sufficient to direct phloem-specific expression of *gus* in transgenic tobacco plants. Expression is observed in phloem sieve tubes and companion cells in roots, stems, petioles, and leaves and is not seen in mesophyll cells or other vascular tissues. The phloem-specific expression directed by this promoter is thus confirmed (Shi et al 1994). Expression levels observed in tobacco were low due to the presence of the first intron of the *RSs1* gene in the 5' untranslated sequence between the transcription start and the translation start.

An alternative strategy to isolate a phloem-specific promoter was also followed by attempting to isolate the promoter from a gene encoding one of the phloem-specific P-proteins. An advantage of these genes is that their products are not selectively accumulated in developing seeds, unlike sucrose synthase. A gene encoding a P-protein was isolated by a protein to cDNA to gene route. Relatively large amounts of phloem exudate from *Cucurbita maxima* (pumpkin) plants were collected and used as a source for purification of the chitin-binding phloem lectin protein designated PP2, a major protein in phloem sap. The partially purified protein was run on SDS-polyacrylamide gel electrophoresis and the most abundant polypeptide was blotted onto PVDF membrane and subjected to protein sequencing. This polypeptide was found to have a blocked N-terminus so, to obtain useful sequence information, the separated polypeptide was cleaved in the gel slice by CNBr and the resulting frag-

ments were purified by reverse phase high-performance liquid chromatography and sequenced. Two fragments were identified. Amino acid sequence data from these polypeptides were used to generate oligonucleotide sequences of lowest redundancy. These were used as probes on a Northern blot of RNA isolated from different organs of developing pumpkin seedlings. Hybridization was observed to an mRNA species of approx. 0.9 kb in RNA from hypocotyls and this tissue was used as a source for cDNA library construction. The library, in the l phage vector ZAPII (Stratagene), was screened with the labeled oligonucleotide and positive plaques were purified. Three clones were fully sequenced. These proved to contain identical PP2 lectin-encoding sequences (Wang et al 1994). The sequence predicted by these clones was in complete agreement with the 78 residues of amino acid sequence determined for the PP2 protein, confirming their identity.

The PP2 cDNA was used as a probe to screen a cucurbit genomic library in the vector l EMBL3 to obtain a gene encoding the PP2 protein. The gene was fully characterized and sequenced. The sequence of the coding region is given in Figure 2. The predicted amino acid sequence in this gene was not identical to that predicted by the cDNA, but encoded a PP2-like protein. PCR of the *C. maxima* seedling cDNA library using primers specific for the gene sequence amplified a fragment of the expected size (data not presented). This result suggests that a cDNA corresponding to the gene is present in the library, and that the gene is highly likely to be expressed. However, when the promoter region was fused to a *gus* reporter gene and the construct was transformed into tobacco plants, no expression of the reporter gene was observed. The reasons for this failure to observe expression are under investigation.

The *RSs1* gene had provided a viable phloem-specific promoter, which was used in subsequent constructs.

Gene constructs

The GNA coding sequence was assembled into two constructs for expression in transgenic plants. A standard transcriptional fusion with the CaMV35S promoter was made for expression in model systems in experiments to "prove" the technology, and a translational fusion between the *RSs1* promoter and the GNA sequence, which introduced the translational start of sucrose synthase and some "linker" amino acids onto the N-terminus of the GNA precursor was made for expression in both model systems and rice.

Testing constructs in a model plant system

Expression in phloem. The *RSs1-GNA* construct was introduced into tobacco via standard *Agrobacterium tumefaciens* transformation procedures. The phloem-specific expression pattern observed with the reporter gene *gus* driven by the *RSs1* promoter was also evident with GNA in the transformed plants. GNA accumulation was determined by immunohistochemical staining (Shi et al 1994) and the presence of GNA in the phloem vessels and companion cells was demonstrated. However, expression levels were indicated to be low, as was found when *gus* was expressed from the *RSs1* promoter in tobacco (see above). The presence of GNA in the phloem sap of these plants

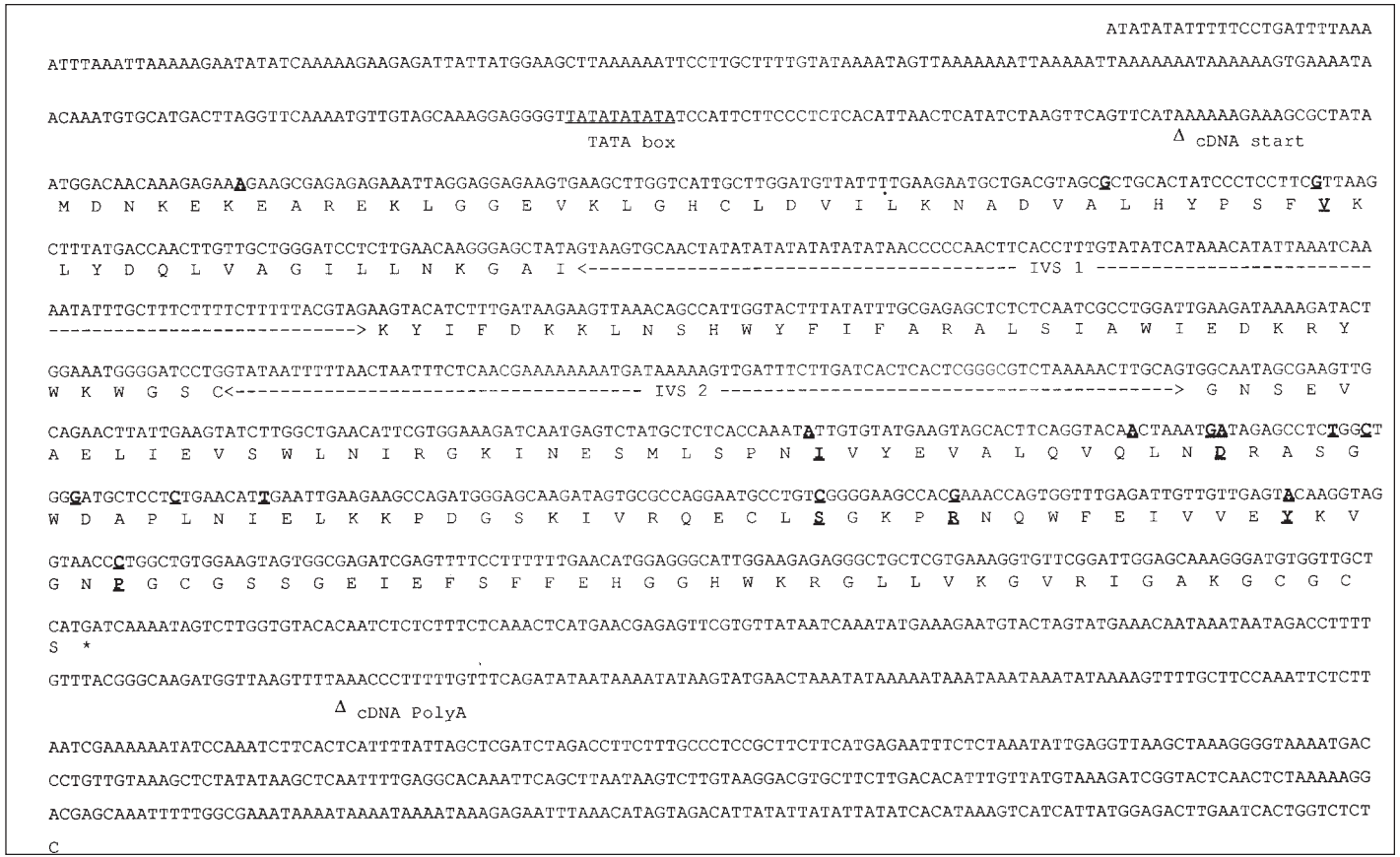

Fig. 2. Sequence of the gene encoding a PP2-like protein from *Cucurbita maxima*. Sequence differences in coding sequence from the cDNA reported by Wang et al (1994) are underlined and in boldface.

was also shown by immunoassay. Peach-potato aphids (*Myzus persicae*) were fed on transgenic and control tobacco plants and the honeydew produced by the aphids was collected on filters. The filters were then processed as immunoblots. This showed the presence of GNA in the honeydew of the aphids feeding on the transgenic plants, but not the control plants. The experiment showed that it was possible to deliver the insecticidal gene product to a sucking insect pest by phloem-specific expression of its encoding gene and proved that the lectin had been transported into the phloem sap from its site of synthesis. The targeting information for this was assumed to have come from the intact GNA leader sequence in the translational fusion construct.

Insecticidal properties. The *CaMV-GNA* construct was introduced into potato using *A. tumefaciens*-based vector systems. Expression of GNA in transformants was measured by dot-blot immunoassay, and was estimated at 0.2-1.0% of total protein, depending on the transgenic line and tissue selected for assay. All work with potato was done on primary transformants, which were vegetatively propagated via shoot cuttings and tubers. Tissue blots showed the presence of GNA in all parts of the plant, but the protein was observed to be selectively accumulated in vascular tissue, and thus would be available to phloem-feeding insects. Potato lines expressing GNA from the CaMV promoter have been subjected to bioassay against lepidopteran and homopteran insect pests, both in the growth room and in the glasshouse. These assays have confirmed that GNA has insecticidal effects and have shown that these effects extend to insect species other than rice pests, although the high level of mortality observed with BPH is not duplicated with more polyphagous pests. Potato plants expressing GNA are protected against attack by larvae of *Lacanobia oleracea*, the tomato moth, with plant damage, larval survival, and larval biomass per plant all significantly reduced. These effects are seen both in growth room and glasshouse bioassays. More relevantly, GNA-expressing potato plants also show resistance to attack by a homopteran pest, the potato aphid *Aulacorthum solani*. In this case, no mortality of insects is observed, but the parthenogenetic production of nymphal offspring is affected, so that the normal population increase of the pest is slowed. Once again, these effects have been observed both in the growth room and in glasshouse trials. Preliminary results showing deleterious effects of GNA expression in transgenic tobacco plants or the peach-potato aphid have been reported (Hilder et al 1994).

These results confirm that GNA expression in transgenic plants is sufficient to confer protection against insect pests, although the degree of protection against polyphagous pests observed in potato is lower than would be expected (or desirable) for transgenic rice exposed to BPH.

Production of transgenic rice

Constructs as described above have been supplied to collaborators in the Rockefeller Foundation Rice Biotechnology Program, who have used the best existing technologies to produce transgenic rice. Both electroporation of protoplasts and the biolistic method, where immature embryos are bombarded, have been used successfully to produce transgenic rice. Details of these technologies are given elsewhere (Hall et al 1993).

Assay of transgenic rice

Putatively transgenic rice plantlets, at the stage where the plantlets have formed root systems, but are still under tissue culture conditions, can be tested for the presence of transgenes by polymerase chain reaction (PCR) on tissue samples. Leaf samples of 0.1 cm^2 can be tested successfully by this method. The technique has been used in Durham to test plantlets for the presence of the GNA gene, using appropriate primers (Fig. 3). To avoid false positive results, control samples must be processed with the

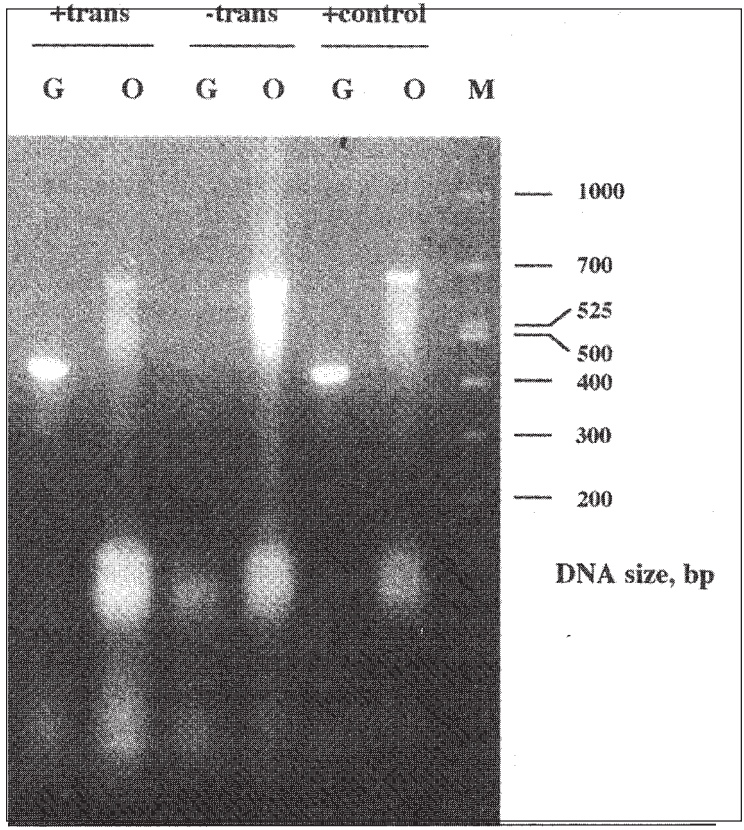

Fig. 3. Assay for presence of GNA coding sequence in putative transgenic rice plants by PCR. G = amplification with GNA primers (expected size of fragment 415 bp); O = amplification with oryzacystatin primers (internal control; expected size of fragment 680 bp); M = size marker calibration. "+ trans" is a transgenic plant containing the GNA coding sequence; "-trans" is a negative transgenic plant; "+control" is a positive control containing approx. 0.1 pg of GNA plasmid DNA. Putative transgenic rice plantlets supplied by M. Davey, University of Nottingham, UK.

putative transgenics; to avoid false negative results, both a control sample spiked with amounts of GNA plasmid (0.1 pg), and amplification of an internal control in the putative transgenics is necessary. The internal control should be a single-copy endogenous rice gene. Amplification of this sequence from the DNA samples extracted from putative transgenic plantlets demonstrates that amplification would work successfully on the transgene as well. Transgenic rice plantlets that contain the GNA transgene have been obtained.

Assay for protein expression using dot-blot, or better, Western blotting techniques using antibodies raised against purified GNA, can be done on rooted plantlets. False positive results have been obtained in dot-blots of rice extracts when reacted with anti-GNA antibodies, and thus Western blotting is necessary to confirm GNA expression. Rice plants transformed with the *RSs1-GNA* construct described above, produced by Prof. Hodges' group at Purdue University, have been assayed for expression of GNA by Western blotting both at Purdue and Durham. Expression levels in the progeny of plants assayed at Durham, raised from seed supplied by Prof. Hodges, have been very low, but some primary transformant plants at Purdue show better expression of levels (T. Hodges, pers. commun.). These plants are being allowed to set seed to provide material for insect bioassays with BPH.

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Notes

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