Invited Paper

Transgenic Rice Endowed with Enhanced Resistance to Major Sap-Sucking Pests

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Abstract

We report, herein, the evolvement of transgenic rice exhibiting resistance to brown planthopper (BPH), green leafhopper (GLH) and whitebacked planthopper (WBPH) by over-expressing mannose-specific lectins of *Allium sativum* agglutinin (ASAL) and *Galanthus nivalis* agglutinin (GNA). Elite *indica* rice cultivars susceptible to different insect pests were transformed using *Agrobacterium*-mediated genetic transformation method. Embryogenic calli derived from mature embryos of rice were co-cultivated with *Agrobacterium* harbouring pSB111 super-binary vector comprising the herbicide resistance *bar* gene along with garlic lectin gene (*asal)* under the control of CaMV35S promoter or snowdrop lectin gene (*gna)* under the regulation of RSs1 promoter. Putatively transformed plants were regenerated from the calli selected on 6-10 mg/l phosphinothricin (PPT)-containing medium. Southern blot analyses confirmed the stable integration of transgenes into the genome of rice plants. Northern and western blot analyses revealed the expression of ASAL and GNA in different transgenic rice lines. In different transformants, the level of ASAL and GNA proteins, estimated by the enzyme-linked immunosorbent assay (ELISA), varied from 0.74% to 1.45% and 0.1% to 0.3% of the total soluble proteins, respectively. Insect bioassays carried out on *asal* and *gna* transgenic rice lines, employing standard screening techniques, revealed high entomotoxic effects of ASAL and GNA on BPH, GLH and WBPH. ASAL- and GNA-expressing transgenics manifested high-level resistance against three major sap-sucking pests and, as such, might serve as a potential genetic resource in recombination-breeding for rice improvement.

Rice is one of the most important cereal crops of the world and serves as the primary source of staple food for more than half of the global population (Emani et al., 2008). Production of rice crop needs to be increased by ~30% to keep pace with the projected population growth (Khush, 2004**).** An approximate 52% of the global production of rice is lost annually owing to the damage caused by various biotic factors, of which ~21% is attributed to the attack of insect pests (Brookes and Barfoot, 2003). Three major sap-sucking pests of rice, *viz*., brown planthopper (*Nilaparvata lugens*, BPH), green leafhopper (*Nephotettix virescens*, GLH) and whitebacked planthopper (*Sogatella furcifera*, WBPH) are known to cause severe damage to rice plants (Dahal et al., 1997; Foissac et al., 2000). These insects cause direct damage to rice plants by sucking the sap and also by plugging xylem and phloem with their stylet sheaths during exploratory feeding. Continuous feeding by the insects results in the drying of crop leading to "hopper burn". Besides causing severe physiological damage to the rice plant, hoppers also act as vectors for rice tungro, grassy stunt and ragged stunt viruses, thereby predisposing the crop to fungal and bacterial infections (Mochida et al., 1979; Saxena and Khan, 1989).

Massive applications of pesticides invariably cause adverse effects on the beneficial organisms besides posing serious risks to the human health and environment. Different major (along with several minor) genes conferring resistance to sap-sucking insects have been identified in the rice germplasm (Bentur et al., 2008). However, the progress made through conventional methods has been slow in evolving insect resistant varieties. Introduction of exotic pest-resistance genes into rice cultivars through molecular

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genetic techniques is an important strategy for achieving increments in the rice productivity, as they provide access to the vast gene pools of diverse taxa (Nagadhara et al., 2003). Transgenic technology is known to offer unique opportunities for effective management of pest populations prevailing in different agro-climatic zones. Introgression of different combinations of resistance genes into the genetic milieu of varieties serves as an effective strategy for achieving durable and broad-based resistance against various insects.

Transgenic plants which express *Bt* endotoxins or protease inhibitors are useful for controlling chewing insects belonging to the order Lepidoptera and Coleoptera (Koziel et al., 1993; Wunn et al., 1996; Nayak et al., 1997; Datta et al., 1998; Khanna and Raina 2002; Ramesh et al., 2004). However, *Bt* transgenics are not effective against the sap-sucking homopteran insects (Rao et al., 1998). Attempts have also been made to produce insect resistant transgenic plants through the introduction of foreign genes encoding plant defensive proteins, such as protease inhibitors (PIs) and lectins with antimetabolic effects (Boulter et al., 1990; Hilder et al., 1987).

Lectins are defined as proteins or glycoproteins of non-immune origin with one or more binding sites per subunit, which can reversibly bind to specific sugar segments through hydrogen bonds and Van Der Waals interactions (Lis and Sharon, 1998). Different plant lectins have been found to show cytotoxic, fungitoxic, anti-insect and anti-nematode properties (Peumans and Van Damme, 1995; Ripoll et al., 2003). Insecticidal activity of the carbohydrate-binding plant lectins against pests belonging to Coleoptera, Diptera, Lepidoptera and Homoptera have been thoroughly investigated (Habibi et al., 1992; Powell et al., 1995; Gatehouse et al., 1995; Carlini and Grossi-de-Sa 2002; Nagadhara et al., 2003, 2004; Majumder et al., 2004; Saha et al., 2006; Sadeghi et al., 2007; Yarasi et al., 2008). Among mannose-binding lectin genes, *Galanthus nivalis* agglutinin (*gna*) has been introduced and expressed in diverse crop plants, *viz.*, rice (Rao et al., 1998; Foissac et al., 2000; Fitches et al., 2001; Nagadhara et al., 2003, 2004), wheat (Stoger et al., 1999), tobacco (Hilder et al., 1995) and potato (Down et al., 1996; Gatehouse et al., 1997; Bell et al., 2001) against different pests. Similarly, *Allium sativum* mannose-specific lectin (*asa* and *asal*) genes have been introduced into rice (Saha et al., 2006; Yarasi et al., 2008) and tobacco plants (Bandyopadhyay et al., 2001; Sadeghi et al., 2007) to provide resistance against homopteran and lepidopteran pests.

This report deals with the development of transgenic rice resistant to major sap-sucking pests by *Agrobacterium*-mediated transfer method employing *asal* and *gna* genes. Molecular evidence confirmed the stable integration of *asal, gna* and *bar* genes in the genome of rice plants. Comprehensive *in planta* insect bioassays demonstrated that ASAL / GNA expression in transgenic lines affords explicit resistance to BPH, GLH and WBPH insects.

Materials and Methods

Transformation studies using pSB111super-binary vectors:

Indica rice cultivars Chaitanya, Phalguna, Swarna and BPT 5204 were used in genetic transformation experiments employing the super-binary vectors pSB11135S *asal*-35S *bar* and pSB111RSs1 *gna*-35S *bar*. Mature seeds were surface sterilized and placed on Murashige and Skoog's (MS) medium (1962), supplemented with 3% sucrose, 2 mg/l 2,4-D, 1 g/l casamino acids, and 0.3% gelrite, for callus induction. The embryogenic callus was cut into 2-3mm pieces and placed on MS medium supplemented with 100µM acetosyringone (AS) for co-cultivation. The *Agrobacterium* culture was grown in YEP medium with 50 mg/l spectinomycin for about 16 h and was transferred to PIM II (Aldemita and Hodges, 1996) containing 100µM AS. After incubation for about 16 h at 29^0C, the bacterial culture was used for co-cultivation of embryogenic calli placed on CCM. At the end of 72 h, calli were washed thoroughly in MS basal medium containing 100 mg/l cefotaxime +250 mg/l carbenicillin and proliferated on MS medium with maltose. Later, the calli (2-3 mm) were subjected to two stages of selection for 3 weeks each on MS media containing 6-8 mg/l and 8-10 mg/l phosphinothricin (PPT). All the media used after co-cultivation up to 2^{nd} stage selection contained 100 mg/l cefotaxime and 250 mg/l carbenicillin. Surviving calli were proliferated on the MS medium devoid of antibiotics and then regenerated on MS medium containing 2 mg/l BAP, 0.5 mg/l NAA, 1.5% sucrose, 1.5% sorbitol and 0.3% gelrite. The regenerated shoots were rooted on the 1/2

strength MS basal medium. The plantlets were transferred to pots and grown to maturity in the glass house. Putative transformants and control (untransformed) plants were tested for the expression of *bar* gene by dipping leaves in 0.25% solution of Basta.

Molecular analysis of putative transgenics

a) PCR analysis : Genomic DNA was isolated from the Basta tolerant and untransformed control plants as per the method (Mc Couch et al., 1988). PCR analysis was carried out using the DNA isolated from the plants that were tolerant to the herbicide Basta. The DNA from the untransformed plants was used as negative control and the intermediate vectors were used as positive controls. For PCR analysis, primers of 5'-CTA CCA TGA GCC CAG AAC G-3' and 5'-TCA GAT CTC GGT GAC GGG-3' for *bar*; 5'-ATG GGT CCT ACT ACT TCA TCT CCT-3' and 5'-TCA AGC AGC ACC GGT GCC AAC CTT-3', for *asal;* and 5'-CGG ATC CAT GGC TAA GGC AAG TCT CCT C-3' and 5'-CGG TAC CTC ATT ACT TTG CCG TCA CAA G-3' for *gna;* were used.

b) Southern and Northern blot analysis : From PCR positive plants, 15µg of genomic DNA was digested with *Sal*I, *Eco*RI and *Hin*dIII enzymes, independently. The digested DNA was resolved on a 0.8% agarose gel and transferred onto positively charged nylon membranes according to Sambrook and Russell (2001). Total RNA was isolated from Southern positive plants and 10-20µg of RNA was used for Northern blot analysis. The coding sequences of *bar, asal* and *gna* were used as radio labeled probes. The labeling reaction was carried out using the random primer labeling beads (Amersham Pharmacia). Pre-hybridization and hybridization reactions were carried out according to Sambrook and Russell (2001).

c) Detection of ASAL and GNA proteins in transformants : Leaf samples of transgenic and untransformed control plants were homogenized in 50 mM Tris-HCl buffer pH 9.0. The extract was centrifuged at 5000 g for 20 min at 4^0C, and the supernatant was collected. Protein samples (5 µg) were subjected to 15% SDS-PAGE and the separated proteins were transferred onto nitrocellulose N-membrane by electroblotting. The membrane was probed with polyclonal rabbit anti-*asal* serum, anti-*gna* serum (1:10000 dilution), and goat anti-rabbit IgG horse-radish peroxidase conjugate as secondary antibody (1:10000 dilution) (Nagadhara et al., 2003; Yarasi et al., 2008).

Wells of the microtitre plate were coated with 1µg of crude protein extract of control and transgenic plants and kept for overnight at 37^0C and at 4^0C for 1 h. The wells were washed thrice with 20mM PBS containing 0.05% Tween 20 and were blocked with 10% non-fat dried milk for 2 h at 37^0C, subsequently washed six times with PBS-T. The primary antibody (1:10000) was added to the wells and incubated for 2 h at 4^0C. After incubation, the wells were washed thrice with PBS and incubated with secondary antibody (1:10000) for 1 h at room temperature. The plates were washed thrice with PBS and 0.001% 3, 3', 5, 5'- tetramethylbenzidine (TMB) substrate in 0.05M phosphate citrate buffer was added along with 0.1% H_2O_2 and kept in dark for 10 min. The reaction was stopped by 1N H_2SO_4 and the absorbance was recorded on ELISA reader at 450nm (Yarasi et al., 2008).

In planta insect bioassays

Insect bioassays were carried out under controlled conditions at Directorate of Rice Research (DRR), Hyderabad. Rice variety (var.) TN1 susceptible to BPH, GLH and WBPH, var. PTB33 resistant to BPH, var. Vikramarya resistant to GLH and var. MO1 resistant to WBPH, besides untransformed plants of cultivars (cvs) Chaitanya and BPT 5204 were used as controls in insect bioassays. The plant progenies of transgenics were transplanted in seed box trays along with respective controls and screened against BPH, GLH and WBPH insects using glasshouse mass screening method (Nagadhara et al., 2003). The susceptible variety (negative control) was planted on either side of the tray i.e., at the boundaries of the seed box trays. The resistant check variety (positive control) was planted in a row in the middle of the tray. The transgenic lines were planted on either side of the resistant check in rows. Ten nymphs per plant of 2nd and 3rd instar stages of BPH, GLH and WBPH were released in to the tray. After fourteen days of infestation with BPH, GLH and WBPH, the surviving plants were scored as resistant, while dead plants were treated as susceptible. The resistance exhibited by transgenic plants was measured based on a scale of 0-9 as used in the International Rice Testing Programme (IRTP, 1980).

Survival and fecundity assays

Thirty-day old homozygous transgenic rice plants (obtained from selfing) and untransformed control plants were used to assess insect mortality /survival in no choice method. Early 1st instar nymphs, 20 each, of BPH, GLH and WBPH were independently released on each plant and confined in an insect proof mylar cage in 10 such replications. Survival was monitored and observations were recorded on the nymphal survival for every 6 day intervals up to 24 days. Further studies were carried out to assess the effect of *asal* and *gna* on fecundity by scoring the nymphs emerged from the eggs hatched. For this study, surviving male and female insects were pooled and confined again in a 1 male: 1 female ratio so that there is no difference in the nymph production based on the sex ratio. A sum of the emerged nymphs and unhatched eggs constituted the fecundity. The total nymphs produced from eggs laid were counted and recorded until no new nymphs were found emerging. Differences in the means of various experiments were assessed and data analysis was done using MS- Excel software and the Sigma plot software version.

Semi-quantitative assay of honey dew production

The extent of insect feeding was estimated by semi-quantitative assay of the honeydew (liquid excreta) produced by the insects (Nagadhara et al., 2003). Whatman No.1 filter paper, dipped in a solution of bromocresol green (2mg/ml in ethanol), was used for honeydew estimation; the filter paper was placed at the base of each plant and covered with a plastic cup. On each plant, five adult female insects of BPH and WBPH, pre- starved for two hours, were released separately and allowed to feed for 24 h. Likewise, honeydew assay was performed for GLH by confining five adult female insects, on a single leaf blade by placing the filter paper at the base of a leaf. Care was taken not to release gravid adult females. Honeydew, excreted by BPH, GLH and WBPH insects, reacts with bromocresol green in the filter paper resulting in blue colour. The area of blue spots developed on filter papers was measured using millimeter graph paper and expressed in units (1 unit=1mm^2). The honeydew stains appeared as violet or purple spots. The spots were traced on tracing paper and squares were counted over a millimeter square graph paper. The area of the honey dew spots was expressed as mm^2 (Nagadhara et al., 2003).

Results

Genetic transformation and production of transgenics

Rice cultivars *viz*., Chaitanya, Phalguna, Swarna and BPT 5204 were transformed using the *Agrobacterium* strain LBA4404 carrying Ti plasmid pSB111-*bar-gna and* pSB111-*bar-asal* (Fig.1). Scutellar-derived calli, after co-cultivation with *Agrobacterium*, were transferred to the Murashige and Skoog (MS) medium containing 6.0-10.0 mg/l phosphinothricin (PPT) as a selection agent. Plants were regenerated from the PPT-resistant calli on the MS medium supplemented with NAA (1.0 mg/l) and BAP (4.0 mg/l). Out of 16184 calli that were co-cultivated, a total of 34 transformants were obtained with pSB111-*bar-gna and* pSB111-*bar-asal* constructs. Leaves from putative (T_0) transformants and untransformed control plants were tested for their tolerance to the herbicide Basta (0.25%). In control plants, Basta treatments caused severe damage to the leaves. Whereas, the leaves of primary transformants retained their normal green appearance and exhibited varied levels of tolerance to the herbicide (Fig. 2).

Confirmation of transgenics at molecular level

Samples of the genomic DNAs, isolated from Basta-tolerant transgenic plants as well as control plants, were tested for the presence of *bar*, *asal* and *gna* genes by PCR using the appropriate primers. PCR analysis showed the presence of ~560bp, ~540bp and ~480bp fragments of *bar*, *asal* and *gna*, respectively, in the genomes of transformants while control plants failed to show such amplification (Fig.3). Genomic DNA digested with *Hin*dIII showed a hybridizable band at, approximately, 1.6 kb with the *bar* probe, and a band at >2.0 kb with the *gna* probe. These bands represent two intact expression units of *bar* and *gna* coding sequences introduced into the genomes of *gna* transgenic plants. When the genomic DNA of transgenic plants was digested with *Hind*III and probed with the *asal* coding sequence, it showed hybridizable band of ~1.6 kb. Similarly, *Eco*RI digested DNA of transgenics probed with the *bar* sequence showed ~1.9 kb band. The untransformed control plants, by contrast, failed

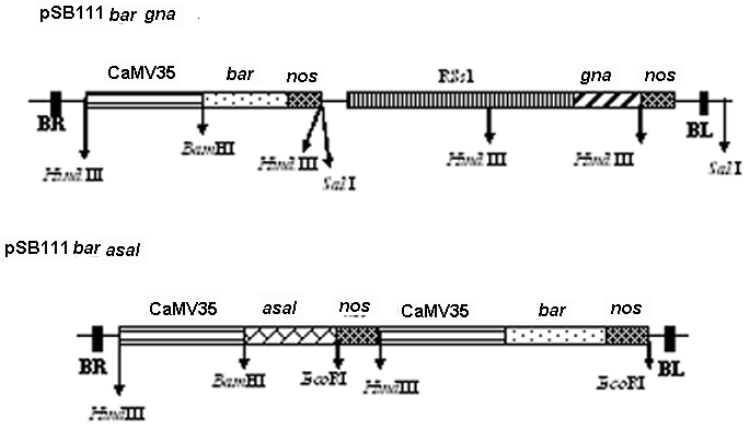

Fig. 1 Restriction map of T-DNA region of pSB111*bar-gna* and pSB111*bar-asal* expression units

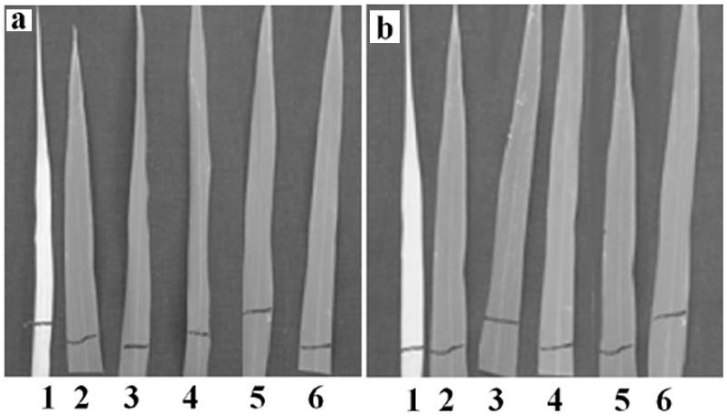

Fig. 2 Basta treated leaves of putative transformants showing complete tolerance to the herbicide

(a) Lane 1: Untransformed Chaitanya control plant showing complete damage to the herbicide Basta; Lanes 2-6: Different *asal* Chaitanya transformants showing herbicide tolerance, (b) Lane 1: Untransformed Chaitanya control plant showing complete damage to the herbicide Basta; Lanes 2-6: Different *gna* Chaitanya transformants showing herbicide tolerance.

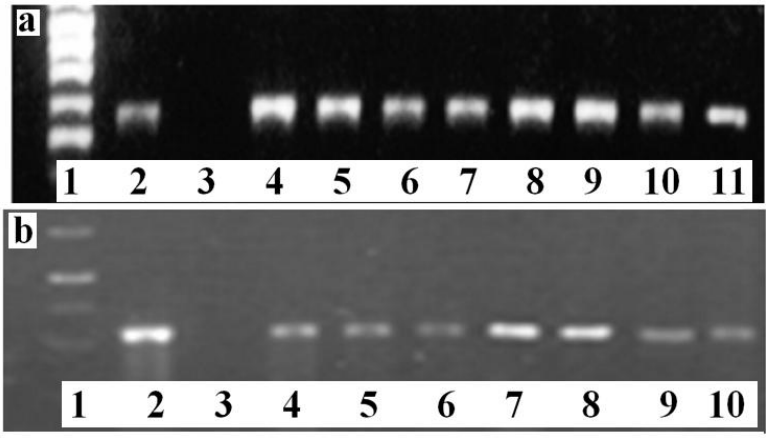

Fig. 3 PCR analysis showing the presence of *asal* and *gna* genes in different rice transformants.

(a) Lane 1: 1kb marker; Lane 2: Positive control showing band corresponding to *asal* gene; Lane 3: Untransformed control showing the absence of band corresponding to *asal* gene; Lanes 4-11: Transgenic lines showing the 546 bp amplicon corresponding to *asal* gene. (b) Lane 1: 1kb marker; Lane 2: Positive control showing band corresponding to *gna* gene; Lane 3: Untransformed control showing the absence of band corresponding to *gna* gene; Lanes 4-10: Transgenic lines showing the 480bp amplicon corresponding to *gna* gene.

to show any hybridizable band when these probes were used.

Northern blot analyses were carried out to observe the expression of *asal* and *gna* genes in the transgenic plants. Northern blots showed the presence of ~1.0 kb and >600bp hybridizable bands with *gna* and *asal* probes, respectively, in different transgenic lines (Fig. 4). Western blot analysis of leaf extracts from transgenic plants showed the presence of ~12 kDa polypeptides corresponding to the purified ASAL and GNA proteins when treated with anti-ASAL and anti-GNA antibodies. The amount of GNA in the transgenic rice plants was estimated to be 0.1 to 0.3% of the total leaf soluble proteins in comparison with the GNA standards on blots, and the amount of ASAL among transformants ranged between 0.74% and 1.45% of the total soluble proteins.

To analyze the inheritance pattern of transgenes, selfed seeds collected from T_0 plants were germinated in pots and T_1 progenies were grown to maturity in the glasshouse. In T_1 and T_2 progenies, both the transgenes *asal* and *gna* co-segregated with the *bar* in a normal Mendelian fashion showing a monogenic ratio of 3 resistant: 1 susceptible plant(s) (Table 1). These transgenic lines were found healthy and were similar to that of untransformed control plants for various morphological characters with normal seed fertility.

Evaluation of transgenic rice lines against sap-sucking pests

Comprehensive *in planta* bioassay experiments were performed to test the insecticidal activity of the *asal* and *gna* genes, on T_1 and T_2 (homozygous) transgenic lines, for three sap-sucking pests of rice. Transgenic rice lines (30-day-old) expressing ASAL and GNA showed significant resistance towards BPH, GLH and WBPH insects with minimal plant damage. Transgenic plants exhibited varied levels of resistance to sap-sucking pests on a par with those of BPH resistant PTB33, GLH- resistant Vikramarya and WBPH resistant MO1, respectively. On the other hand, the susceptible TN1 and untransformed control plants showed complete damage (9 score on a 0-9 scale) caused by these insects (Fig.5).

The selected transgenic lines were further subjected to insect bioassays for mortality, developmental delay, fecundity and feeding behaviour of insects. ASAL- and GNA-expressing plants from selected lines, when infested with BPH/GLH/WBPH nymphs, survived the infestation and could grow to maturity with normal seed set. The survival of BPH, GLH and WBPH nymphs fed on transgenic rice plants was reduced by 74%, 79% and 64%, respectively, compared to that of susceptible control plants. During the entire 24-day bioassay period, the survival of BPH on transgenic plants was significantly reduced to 3.5±1.1 insects/plant on ASAL- transgenic line and 6.0±1.0 insects on GNA transgenic line, compared to 12.4±1.3 insects/ plant observed on untransformed control plants. Likewise, the survival of GLH on transgenic rice was significantly reduced to 2.4±1.1 insects/plant on ASAL- transgenic line and 6.1±0.9 insects on GNA- transgenic line, compared to 11.4±1.3 insects/plant on control plants. Furthermore, the survival of WBPH fed on transgenic plants was reduced to 4.7±1.1 insect/plant on ASAL-transgenic line and 2.1±1.1 insects on GNA-transgenic line, in comparison with 11.9±1.1 insects/plant recorded on control plants (Fig. 6).

Effect of ASAL and GNA on the fecundity of BPH, GLH and WBPH was assessed by estimating the total number of nymphs produced by the insects fed on transgenic rice plants. A mean number of 103±3.9 BPH nymphs/plant were observed on ASAL- transgenic plants and 165.0±3.2 nymphs on GNA- transgenic plants compared to 315 ± 4.5 nymphs/plant found on untransformed controls. In case of GLH, a mean number of 112±1.4 nymphs/plant was observed on ASAL- transgenic line and 170.0±5.1 nymphs were noted on GNA- transgenic plants in comparison with 348±5.01 nymphs/plant on control plants. Similarly, for WBPH a mean number of 104±1.55 nymphs/plant was noticed on ASAL- transgenic line and 50.0±4.6 nymphs on GNA- transgenic plants compared to 380±5.2 nymphs/plant produced on untransformed controls (Fig. 7).

The feeding ability of insects was assessed based on the amount of honeydew excreted by the insects. After a lapse of 24 h of feeding on transgenic rice/ untransformed control plants, the number of honeydew units (blue spots) developed on the bromocresol green paper was

Table 1: Inheritance pattern of transgenes in T_1 generation.

Progeny	Test/ Bioassay	No. of plants resistant	No. of plants susceptible	Segre-gation ratio	χ 2 value	*p*-value
Herbicide tolerance						
A1*	Basta	21	8	3:1	0.096	0.756
A2*	Basta	23	12	3:1	1.609	0.204
A3*	Basta	24	11	3:1	0.771	0.379
A4*	Basta	24	10	3:1	0.359	0.555
A5*	Basta	26	10	3:1	0.148	0.700
A6*	Basta	17	9	3:1	1.282	0.257
A7*	Basta	19	9	3:1	0.761	0.382
A8*	Basta	21	8	3:1	0.096	0.756
A9*	Basta	29	12	3:1	0.380	0.537
G1**	Basta	19	6	3:1	0.013	0.909
G2**	Basta	21	8	3:1	0.096	0.756
G3**	Basta	37	13	3:1	0.026	0.871
G4**	Basta	29	12	3:1	0.380	0.537
G5**	Basta	29	11	3:1	0.130	0.718
G6**	Basta	29	7	3:1	0.273	0.601
G7**	Basta	20	7	3:1	0.012	0.912
G8**	Basta	36	13	3:1	0.055	0.814
G9**	Basta	39	15	3:1	0.216	0.642
Insect bioassays						
A1*		26	9	3:1	0.009	0.922
A2*	BPH	29	10	3:1	0.059	0.808
A3*	BPH	23	8	3:1	0.010	0.920
A4*	BPH	32	11	3:1	0.007	0.933
A5*	GLH	23	8	3:1	0.010	0.920
A6*	GLH	30	10	3:1	0.130	0.718
A7*	GLH	32	11	3:1	0.007	0.933
A8*	WBPH	22	8	3:1	0.041	0.839
A9*	WBPH	36	13	3:1	0.055	0.814
G1**	WBPH	37	13	3:1	0.026	0.871
G2**	BPH	28	10	3:1	0.036	0.849
G3**	BPH	29	10	3:1	0.059	0.808
G4**	BPH	31	10	3:1	0.380	0.537
G5**	GLH	34	12	3:1	0.028	0.867
G6**	GLH	29	9	3:1	0.036	0.849
G7**	GLH	27	9	3:1	0.273	0.601
G8**	WBPH	29	10	3:1	0.059	0.808
G9**	WBPH	34	12	3:1	0.028	0.867
	WBPH					

* ASAL transgenic lines; ** GNA transgenic lines

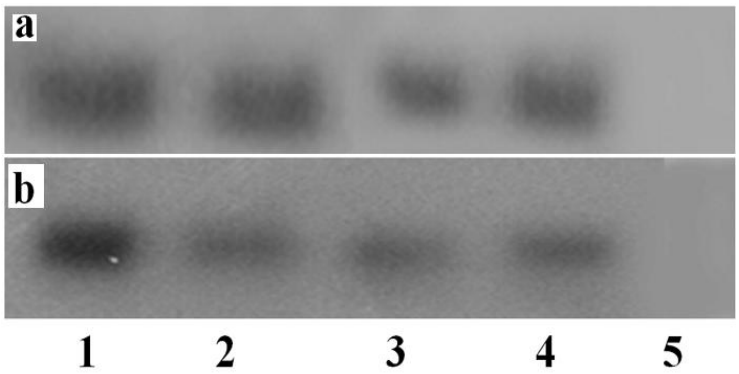

Fig. 4 Northern blot analysis for the expression pattern of *asal* and *gna* genes in different transgenic rice lines.
(a) RNA blot probed with *asal* coding sequence. (b) RNA blot probed with *gna* coding sequence. Lanes 1-4: RNA from transgenic lines of Chaitanya; Lane 5: RNA from untransformed control plant.

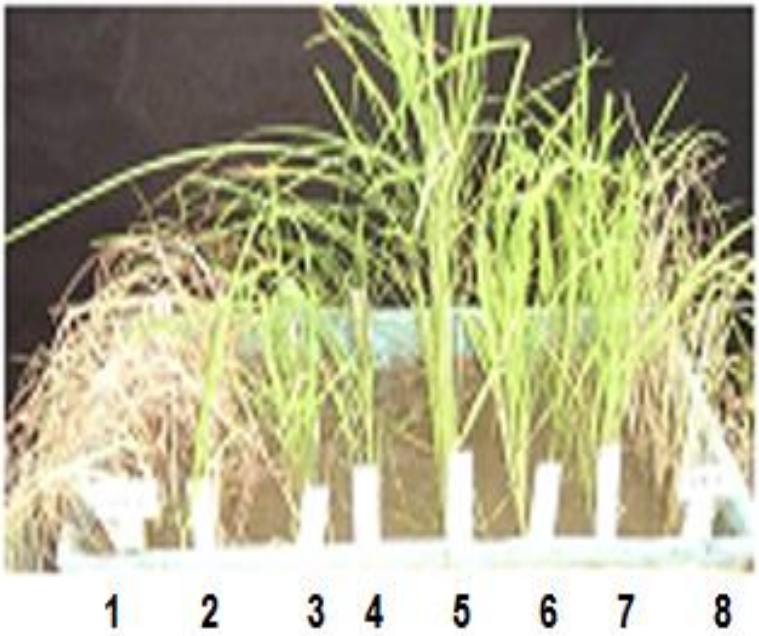

Fig. 5 Insect bioassays on T_2 homozygous transgenic plants of Chaitanya

30-day old transgenic lines along with controls infested with BPH. Rows 1 and 8: Control (var.TN-1) plants showing complete damage; Row 2: Untransformed Chaitanya control plants showing damage; Rows 3 and 6: ASAL transgenic lines showing resistance against BPH; Row 5: Resistant check (var. PTB33) for BPH; Rows 4 and 7: GNA transgenic lines showing resistance against BPH. Photographs were taken after 14 days of infestation.

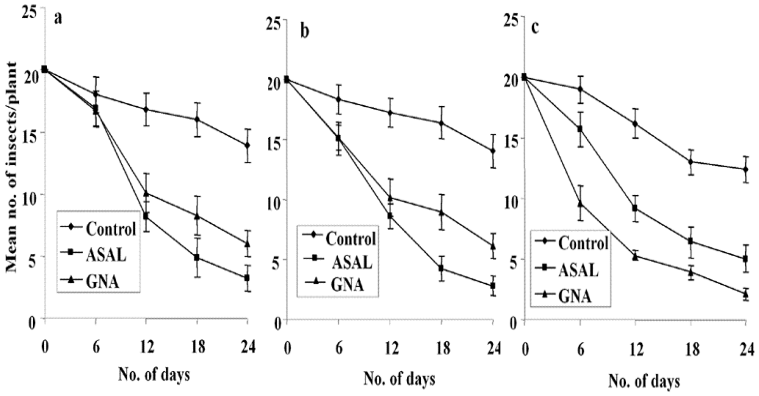

Fig. 6 Survival of BPH, GLH and WBPH insects on transgenic rice lines expressing ASAL and GNA.
Twenty 1st instar nymphs of BPH (a), GLH (b) and WBPH (c) were released on each plant on day 0. Homozygous GNA transgenic lines are depicted by triangle, homozygous ASAL transgenic lines are depicted by square. Control plants are depicted by diamond. Bioassays were carried out on 20 plants sampled from each transgenic line and two controls. Bars indicate mean ±SE.

counted to estimate the feeding capacity of the insects. A mean number of 8±1.36, 21±2.12 and 25±4.06 honeydew units/ plant on ASAL-transgenic plants and 16.0±2.3, 21.0±1.2 and 15.0±1.4 units/plant on GNA transgenic lines were produced by BPH, GLH and WBPH, respectively, compared to 162±6.7, 173±6.32 and 189±7.3 honeydew units/plant observed on control plants (Fig. 8).

Discussion

Plant lectins, such as GNA, Con A, PSA and ASA exhibiting mannose or mannose/glucose sugar binding affinity, revealed palpable anti-metabolic effects towards members of the homopteran insects both under *in vitro* and *in planta* conditions (Powell et al., 1993; Rahbe et al., 1995; Powell et al., 1995; Gatehouse et al., 1996). Although the mode of action of plant lectins is not well understood, it appears that their resistance to proteolytic degradation by the insect digestive enzymes and binding to gut structures are essential for lectins to exert their deleterious effects on insects (Pusztai, 1991; Carbonaro et al., 1997; Gatehouse et al., 1984; Bandyopadhyay et al., 2001). Employing the protocols optimized in our laboratory (Ramesh et al., 2004), the co-integrated super-binary vectors, pSB111-*bar-asal* and pSB111-*bar-gna*, have been used to transform elite *indica* rice cultivars. PCR and Southern analyses of BASTA tolerant plants confirmed the stable integration of *bar*, *asal* and *gna* genes in the rice genome. Presence of ~1.6 kb hybridizable band with the *asal* probe in *Hind*III digested DNA, ~1.9 kb hybridizable band with the *bar* probe in *Eco*RI digested DNA (Yarasi et al., 2008), and appearance of ~1.6 kb hybridizable band with the *bar* probe, >2.0 kb band with the *gna* probe in the *Hin*dIII digested genomic DNA (Nagadhara et al., 2003) of transformants establish the presence of intact expression units of *bar-asal* and *bar-gna* in the rice genome. Northern blot analysis disclosed variable expression of *asal* and *gna* genes in the primary transgenic plants as evidenced by the varied intensity of hybridizable bands (Fig. 4). Marked variations observed in the amount of ASAL (0.74% to 1.45%) and GNA (0.1% to 0.3%) proteins in different transformants, as revealed by ELISA analysis, testify that the transgenes are integrated randomly at different transcriptionally active sites in the rice genome.

For establishing the transgenic nature of primary transformants, the inheritance pattern of transgenes was analyzed in the T_1 generation. Segregation analyses of transgenes in T_1 progenies revealed a monogenic ratio of 3 resistant: 1 susceptible plant(s) for both herbicide tolerance and insect resistance, affirming that these genes are stably integrated into the rice genome. The co-segregation of transgenes further confirms that both *bar* & *asal* and *gna* & *bar* are co-integrated and manifest as a single locus.

To control phloem-feeding vectors and vector-mediated transmission of plant viruses, phloem-specific expression of genes is essential as it directly affects the phloem-feeding target insects and avoids unwarranted expression in nontarget organs and tissues, thereby minimizing the metabolic load on the transgenic plants. The use of phloem-specific promoters to express GNA in the phloem tissues of transgenic plants not only confers resistance against sap-sucking insects but also prevents the accumulation of GNA in other plant parts (Nagadhara et al., 2004). Therefore, the rice sucrose-synthase-gene (RSs1) promoter was utilized to drive the expression of *gna* in the phloem tissues of rice plants.

T_2 progenies of homozygous transgenic lines, subjected to insect bioassays, exhibited high-level (1-2 score on a 0-9 scale) resistance (Fig.5). After 24 days of infestation, insects surviving on transgenic plants varied from 2 to 4/plant. The survival of BPH was reduced by 74% on ASAL transgenic lines and by 55% GNA transgenic lines as compared to the control plants. Similarly, GLH survival was decreased by 79% on ASAL transgenic lines and by 49% on GNA transgenic lines when compared to the susceptible controls. However, the survival of WBPH was declined by 64% on ASAL transgenic lines and by 90% on GNA transgenic lines in comparison with susceptible control plants (Fig. 6). Furthermore, fecundity assays conducted on ASAL- and GNA- transgenic lines revealed significant decline in the nymphal production of BPH, GLH and WBPH insects by 68%, 73% and 67% on ASAL- transgenic lines and by 53%, 51% and 90% on GNA- transgenic plants, respectively, indicating marked decreases in the fecundity of these insects (Fig.7). These results amply suggest the high antifeedant and entomotoxic effects of ASAL and GNA on major sucking insects. Also, marked decreases of 92 % & 80 %, and 80% & 69% were observed in the honeydew production of BPH and GLH

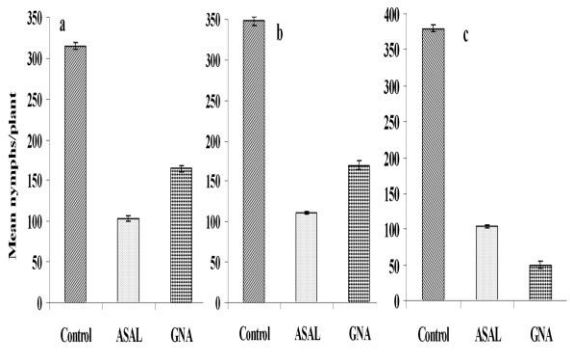

Fig. 7 Effect of ASAL and GNA on the fecundity of sucking insects.
Total number of nymphs produced by a pair of adult BPH (a), GLH (b) and WBPH (c) insects on controls and transgenic plants were counted and were plotted on the graph. Bioassays were carried out on 20 plants sampled from each transgenic line and controls. Bars indicate mean±SE.

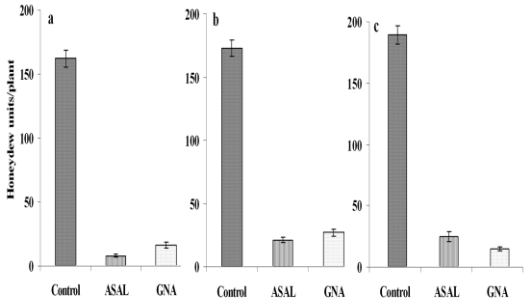

Fig. 8 Effect of transgenic plants expressing ASAL and GNA on feeding behaviour of sucking insects.

a). Semi-quantitative estimation of honeydew excretion by BPH insects. b). Semi-quantitative estimation of honeydew excretion by GLH insects. c). Semi-quantitative estimation of honeydew excretion by WBPH insects. Bars indicate mean ±SE

insects, respectively, when fed on ASAL- and GNA- transgenic plants, respectively. Conversely, GNA-transgenics showed 90% reduction in the honeydew production of WBPH compared to 60% reduction observed on ASAL-transgenics (Fig. 8). *In planta* insect bioassays demonstrated that expression of ASAL and GNA in transgenic rice lines impart substantial resistance against BPH, GLH and WBPH insects owing to decreased insect feeding and declined insect survival, thereby minimizing the damage caused by hopper burn.

Although the precise mechanism of lectin toxicity to insects is unclear, yet it probably involves binding of lectins to the receptors present on the gut epithelial cells of various insects (Harper et al., 1995). Immuno-histochemical studies of a wide range of mannose or mannose/glucose specific lectins such as GNA, Con A and PSA suggest the binding to the midgut epithelial cells of insects thereby contributing to the insecticidal effect (Powell et al., 1998). Furthermore, the bound lectins might inhibit the absorption of nutrients or disrupt the midgut cells through endocytosis of lectin and other toxic metabolites (Eisemann et al., 1994). The toxicity of mannose-binding lectins towards sucking insects is not clear, but it has been shown to bind to the mannose moiety of brush-border-membrane vesicle (BBMV) receptors of gut epithelial cells, resulting in the disruption of cell function and insect mortality (Bandyopadhyay et al., 2001; Fitches et al., 2001). The variable entomotoxic effects of GNA and ASAL on three sap-sucking pests are attributable to their differential binding affinities to the receptor proteins on the gut epithelial cells of insects.

To sum up, the ASAL and GNA expressing rice lines, bestowed with high antifeedant and antimetabolic effects, afforded high-level resistance against major sap-sucking insects. The overall results substantiate that ASAL is more toxic to BPH and GLH insects when compared to GNA; whereas, GNA showed higher toxicity to the WBPH than that of ASAL under similar insect bioassay conditions. The prototypic *asal* and *gna* transgenic rice lines appear promising for direct commercial cultivation, besides serving as a potential genetic resource in recombination-breeding for insect resistance.

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