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ORIGINAL RESEARCH PAPER

Phloem-specific expression of the lectin gene from *Allium sativum* confers resistance to the sap-sucker *Nilaparvata lugens*

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Abstract Rice production is severely hampered by insect pests. Garlic lectin gene (*ASAL*) holds great promise in conferring protection against chewing (lepidopteran) and sap-sucking (homopteran) insect pests. We have developed transgenic rice lines resistant to sap-sucking brown hopper (*Nilaparvata lugens*) by ectopic expression of *ASAL* in their phloem tissues. Molecular analyses of T_0 lines confirmed stable integration of transgene. T_1 lines (NP 1-2, 4-3, 11-6 & 17-7) showed active transcription and translation of *ASAL* transgene. ELISA revealed ASAL expression was as high as 0.95 % of total soluble protein. Insect bioassays on T_2 homozygous lines (NP 18 & 32) revealed significant reduction (~74–83 %) in survival rate, development and fecundity of brown hoppers in comparison to wild type. Transgenics exhibited enhanced resistance (1–2 score) against brown hoppers, minimal plant damage and no growth penalty or phenotypic abnormalities.

Keywords Brown plant hopper · Garlic lectin · Phloem-specific promoter · Plant hopper insect · Rice · Sap sucker insect · Transgenic rice

Introduction

Production of rice (*Oryza sativa* L.) is hampered by several pests and diseases. Among the insect pests, sap-sucking brown hopper (*Nilaparvata lugens*), the green leafhopper (*Nephotettix virescens*) and the white-backed plant hopper (*Sogatella furcifera*) are the most devastating, causing huge losses every year (Brookes and Barfoot 2003). These plant hoppers cause damage to rice by removal of phloem sap and production of 'hopper burn' in crops by blocking phloem vessels. In addition, plant hopper act as vectors for transmission of tungro grassy, and ragged stunt viruses. Arresting their survival and growth by systemic and contact insecticides is ineffective due to their ability to acquire resistance to these chemicals (Gallagher et al. 1994). Moreover, insecticides are expensive, weather-dependent and exert an adverse impact on the environment and human health.

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Alternatives, such as biological control and introduction of endogenous plant hopper resistant rice genes into rice cultivars through conventional breeding, have shown some success. Incidentally, the plant hopper resistance trait was unstable due to evolution of resistance breaking biotypes in rice (Gallagher et al. 1994).

Genetic engineering of lectins in important crops is an effective and viable strategy to reduce survival, development and fecundity of sap-sucking insects and thereby can reduce crop damage. Among various lectins, the snowdrop [*Galanthus nivalis* L. agglutinin (GNA)] and garlic [*Allium sativum* L. (ASAL)] lectins have a detrimental effect on plant hoppers. Snowdrop (*G. nivalis*) lectin gene (gna) has been expressed in diverse crop plants (Nagadhara et al. 2003, 2004; Couty et al. 2001) to confer protection against different sucking pests. Similarly, expression of garlic lectin encoding genes (*ASA* and *ASAL*) in rice (Saha et al. 2006; Yarasi et al. 2008) and cotton (Vajhala et al. 2013) conveyed marked resistance against homopteran and lepidopteran pests. *ASAL* and *GNA* pyramided rice lines showed higher degree of resistance against sap-sucking insect families in comparison to those that express individual genes (Bharathi et al. 2011).

Although several reports exist on developing rice resistance to brown hoppers very few have shown any efficacy against them. The present study involves the phloem-specific (under control of RSs-1 promoter) expression of garlic leaf lectin in transgenic japonica (Nipponbare-a brown plant hopper susceptible variety) rice lines to confer brown hopper resistance. Molecular characterization, ELISA and immunohistochemical assays of transgenic lines were performed. Bioassays of T_2 transgenic rice lines were done to monitor the percentage survival, development and fecundity of brown plant hopper (*N. lugens*) in comparison to control plants.

Materials and methods

Plant material and bacterial strains

Japonica rice seeds and brown hoppers were procured from Directorate of Rice Research, Hyderabad, India. *E. coli* DH-5α and *Agrobacterium tumefaciens* LBA 4404 were used for cloning and plant transformation, respectively. Brown plant hoppers (*N. lugens*) were maintained on TN1 (cultivar Taichung Native 1, a susceptible variety) rice plants (30 days old), under controlled greenhouse conditions.

Binary vector construction, rice transformation and regeneration of T_0 *ASAL* transgenic plants

Expression cassette of *ASAL* gene (U58947) driven by a rice phloem-specific promoter (AJ401233) was cloned in multiple cloning sites of the entry vector-1 (EV-1). The *ASAL* cassette was transferred to pMDC99 vector by site-specific homologous recombination and was designated as pMDC99-*ASAL* (pMDC99-RSs1: *ASAL: 35s*-PolyA) (Fig. 1a). The plasmid of recombinant pMDC99 harbouring the *ASAL* cassette was mobilized into *Agrobacterium* for transformation of rice. Scutellum-derived embryogenic calli from mature dry rice seeds (var. Nipponbare) infected with the recombinant *A. tumefaciens* were selected on hygromycin (50 mg/l) containing MS medium (B5 vitamins) with 2,4-D (2.5 mg/l), BAP (0.3 mg/l), carbenicillin (250 mg/l) and cefotaxime (250 mg/l) for 40 days. Regenerated T_0 generation plantlets on MS (B5 vitamins), containing TDZ (0.5 mg/l), BAP (3 mg/l) and NAA (0.2 mg/l), were transferred onto the half-MS rooting medium. Rooted plants were transferred to pots and grown to maturity in the greenhouse maintained under standard conditions (Mallikarjuna et al. 2011).

Molecular analyses of transgenic lines

Genomic DNA was isolated from four putative T_0 hygromycin resistant lines (NP 1, 4, 11 and 17) and control plants using CTAB buffer for PCR detection of *hpt* and *ASAL* genes in these lines. PCR was carried out using the following primers (hptF5′-GCCTGAACTCA CCGCGACG-3′, hptR 5′-CAGCCATCGGTCCAG ACG-3′; *ASAL*-F 5′-ATGGGCAGGACCACCTCTT C-3′, *ASAL*-R 5′-CGGTCACATTCTGGTTCAC-3′), respectively in a PCR mixture (50 µl) containing template gDNA (200 ng), primers (150 ng), buffer (1×), dNTPs (0.2 mM) and *Taq* DNA polymerase. The PCR conditions were 94 °C, 1 min; 56 °C, 1 min and 72 °C, 1 min for 30 cycles. Integration of T-DNA in the rice genome was analysed by PCR amplification of the *ASAL* transgenic and *hpt* expression cassettes and further scored for plants with PCR positive amplification.

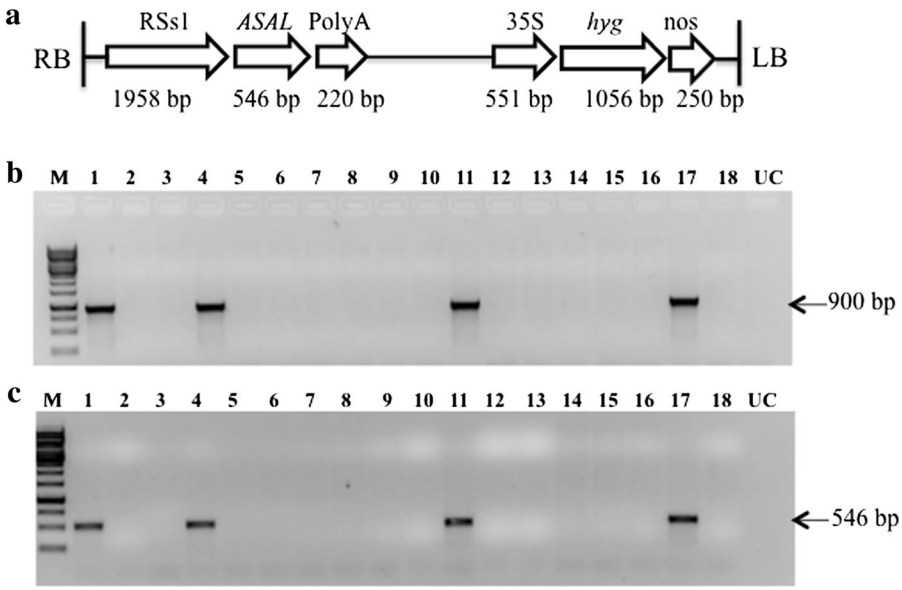

Fig. 1 **a** Schematic representation of the T-DNA region of the *ASAL* construct in plant transformation vector pMDC99. **b** Putative T_0 transgenic plants show amplification of hyg^r sequence showing ~900 bp. **c** Putative transgenic plants show amplification of *ASAL* gene showing ~546 bp. (*lane M* 1 kb marker; *lane C* Genomic DNA from control plant; *lanes 1–18* genomic DNA from different putative transformants)

For Southern analysis, genomic DNA (20 µg) from PCR positive T_0 lines was digested with *Spe*I, separated on 0.8 % agarose gel, electrophoresed and transferred to a nylon membrane. The blot was hybridized with 546 bp-*ASAL* coding region as probe, which was prepared by the PCR DIG probe synthesis kit (Roche Diagnostics). Membrane washing and detection procedures were performed according to the supplier's instructions.

Putative T_0 transgenic plants (NP-1 and 11) were used to isolate genomic DNA for identification of the gene integration locus by directional genome walking (Reddy et al. 2002). The amplified PCR products were cloned and sequenced. Sequence integration in the genome was confirmed by BLAST analysis.

Seeds of T_1 progenies (NP 1-2, 4-3, 11-6 and 17-7) were germinated on growth medium containing hygromycin (30 mg/ml) to monitor the segregation pattern of *HPT* gene. PCR analysis of the hygromycin-resistant progeny was performed to identify the presence of *HPT* and *ASAL* transgenes. For northern analysis, total RNA (20 µg) was isolated from 30 day old hygromycin-selected and PCR positive T_1 rice lines and control using Trizol. RNA was run on denaturing agarose gel containing formaldehyde and transferred to a nylon membrane. The blot was hybridized with *ASAL* coding region (546 bp) as probe, which was prepared by PCR DIG Probe synthesis kit (Roche, Switzerland) following supplier's instructions. Membrane washing and detection procedures were performed according to the supplier's instructions.

Recombinant ASAL was expressed in *E.coli* by cloning the full-length coding sequence of garlic *ASAL* was in *Nde*I and *Not*I sites of pET-28a expression vector (Novagen, USA). Recombinant protein was purified to homogeneity using nickel—NTA (Qiagen, Germany) column following the manufacturer's instructions. Polyclonal antibodies were developed against ASAL protein by immunizing rabbits (New Zealand) with purified recombinant ASAL protein. Western analysis was carried out using total leaf proteins from 1-month-old T_1 transgenic and control plants. These proteins were fractionated on a 12 % (v/v) SDS-PAGE and electroblotted onto a nitrocellulose membrane. Detection of ASAL polypeptide by anti-ASAL polyclonal antibodies was carried out as described (Towbin et al. 1979). Quantitative levels of ASAL expression in T_2 transgenics were estimated by ELISA according to Saha et al. (2006).

Insect bioassays

Seeds of T_1 and T_2 progenies were germinated and grown under controlled greenhouse conditions. These transgenic plants (30 days old) were subjected to insect bioassays to assess insect feeding and plant damage in comparison to control plants in the greenhouse under controlled conditions. Early 1st

instar nymphs of brown hopper were released into the insect proof mylar cage to feed on T_2 progenies (30 days old) and control plants of the same age for 30 days. Nymph distribution and their survival on different plants at 2 day intervals for 30 days were recorded. The resistance exhibited by transgenic plants was also scored on a scale of 0–9 as used in the International Rice Testing Programme (1980).

Results

Rice transformation, inheritance of transgenes and molecular characterization of transgenic lines

Plant transformation construct comprised of the complete coding sequence of the synthetic garlic leaf lectin (ASAL) gene codon optimized for expression in rice. The construct was designed as a transcriptional fusion between the phloem-specific rice sucrose synthase promoter (RSs1) along with 5′-untranslated region of rice sucrose synthase and the coding sequence of the garlic leaf lectin (ASAL). Construct was designed to express garlic lectin in phloem tissue to confer resistance to sap-sucking insects, thereby maximizing expression of the insecticidal protein at the site of attack in the plant (Fig. 1a). Plants were regenerated from hygromycin resistant calli (see "Materials and methods" section).

Out of 200 co-cultivated calli, 18 hygromycin-tolerant putative transgenic plants were obtained. Samples of genomic DNA isolated from hygromycin-tolerant putative transgenic plants as well as control plants were analyzed. PCR analysis using the appropriate primers showed the presence of a 900 bp fragment of *HPT* and a 546 bp fragment of *ASAL* in four transgenic plants (NP1, 4, 11 and 17) out of 18 (Fig. 1b, c). Southern analysis of PCR-positive T_0 transgenic rice plants (NP1, 4, 11 and 17) revealed a distinct hybridized band showing single copy nature at different places of genome that was absent in the control or control plants (Fig. 2a). Selected transgenic plants NP 1 and NP 11 showed ASAL gene integration loci on chromosome 12 and 9, respectively (Fig. 2b, c). Results confirmed that the two transgenic lines NP 1 and NP 11 were different transgenic events.

To investigate the inheritance pattern of the transgenes, T_1 seeds collected from T_0 plants were germinated on MS medium containing hygromycin (30 mg/l) and scored for the number of seedlings grown. Further T_1 progeny (NP 1-2, 4-3, 11-6 and 17-7) were tested for the presence of *ASAL* sequence by PCR. Both the hygromycin resistance and *ASAL* co-segregated in a normal Mendelian fashion, and showed a monogenic ratio of 3 resistant:1 susceptible (Supplementary Fig. 1; Table 1). Northern and Western blot analyses were carried out to observe the expression of *ASAL* gene in T_1 transgenic plants (NP 1-2, 4-3, 11-6, 17-7) screened by hygromycin germination test. Northern blots showed the presence of a 1 kb-hybridized band with the *ASAL* probe; however, no such band was observed in the control or control plants (Fig. 3a, b). Western blotting of leaf extracts from transgenic plants showed the presence of a polypeptide of $\sim$12 kDa that corresponded to the expected ASAL protein. However, no such protein was observed in the control or control (Fig. 3c). These results suggested the active transcription and translation of *ASAL* gene in T_1 transgenic lines.

The rice transgenic and control plants were assayed for accumulation of ASAL in stem tissues. The in situ immunochemical staining of stem transverse section from T_2-NP 18 transgenic plant detected the presence of ASAL only in phloem tissues, whereas there was no detection of ASAL in control section (Supplementary Fig. 1). Results demonstrated that the tissue specific expression of ASAL under the rice sucrose synthase-1 promoter (RSs1) in transgenic lines.

Entomotoxic effects of ASAL on survival, development and fecundity of brown hoppers feeding on T_1 and T_2 lines

Feeding experiments for brown hoppers on T_1 transgenic (NP 1-2, NP 4-3, NP11-6 and NP 17-7) and control rice plants (30 days old) were carried out with first instar nymphs over 30 days. During the first 5 days of the screen, the distribution of brown hopper nymphs was insignificantly different between ASAL expressing transgenic rice plants and control. Brown hopper nymphs were unable to differentiate between the transgenic and control plants. However, from the sixth day onwards, the number of brown hopper nymphs on the transgenic ASAL expressing plants progressively decreased in comparison to the control. A gradual decline in the insect population feeding on transgenic lines was observed, when given a choice to feed on either transgenic or control. Reduction in brown hopper population directly correlated with the

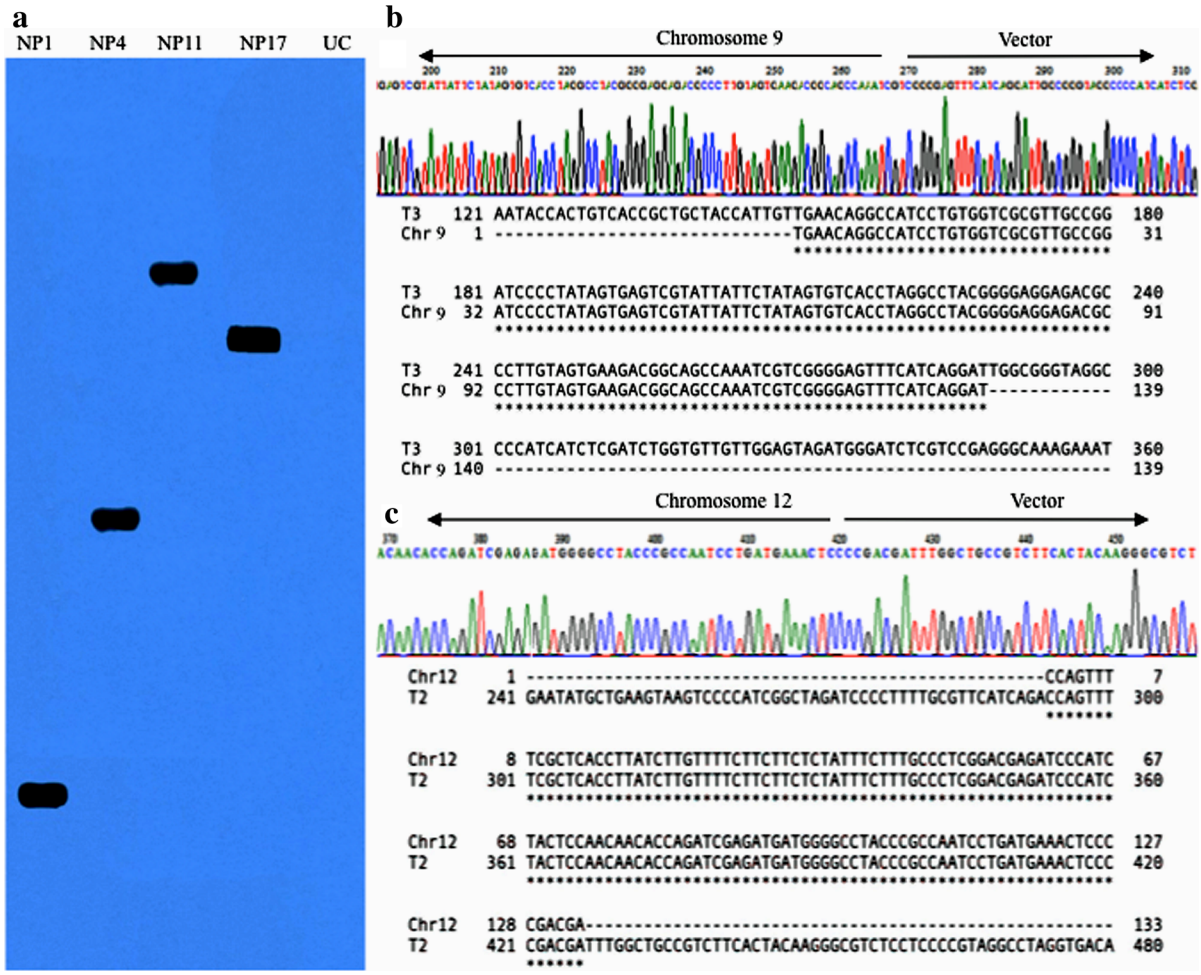

Fig. 2 **a** Southern analysis of T_0 transgenic rice and control plants. *Blot* shows *Spe*I digested genomic DNAs probed with *ASAL* coding sequence. [*Lanes NP 1*, *NP 4*, *NP 11* and *NP 17* represent different transgenic lines. *Lane UC* represents control plant]. Transgenic lines show *ASAL* Integration in chromosome number 12 for NP 1 (**b**) and 9 for NP 11 (**c**)

degree of brown hopper resistance developed in *ASAL* transgenic lines. The ASAL expression led to subsequent reduction in plant damage due to brown hoppers in comparison to the damage caused in control plants (Fig. 4). Brown plant hopper-induced plant damage observed among the ASAL expressing T_1 seedlings was heterogeneous, probably due to the segregating status of the transgene. Transgenic rice lines (30 days old) expressing ASAL showed significant resistance towards brown hopper insects with minimal plant damage (Fig. 4). Transgenic plants exhibited varied levels (1–2 score on a 0–9 scale) of resistance to brown hoppers. On the other hand, control plants of susceptible rice varieties (TN1 and Nipponbare) showed complete damage (i.e., scored 9 on a scale of 0–9) caused by these insects (Supplementary Table 1).

Amongst several T_2 transgenic lines tested, two lines NP 18 (from T_1-NP 1-2) and NP 32 (from T_1-NP 11-6) of Nipponbare showed higher levels of resistance. The selected transgenic lines were further subjected to insect bioassays for monitoring the mortality, developmental delay, fecundity and feeding behaviour of insects. These ASAL expressing transgenics infested with brown hopper nymphs survived the infestation and could grow to maturity with normal seed set. The survival of brown hopper was reduced by ~74–83 % when fed on ASAL transgenic plants in comparison to those fed on susceptible control plants (Fig. 5a). During the entire bioassay (30 days), the

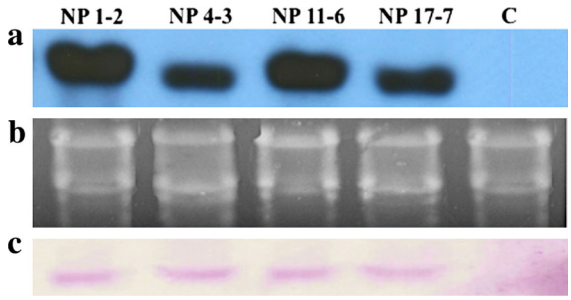

Fig. 3 Comparative expression analyses of *ASAL* at **a** transcript and **c** protein levels in transgenic and control rice lines. **a** Northern blot shows hybridized bands for *ASAL* in T_1 transgenic lines of japonica (*lanes NP 1-2*, *NP 4-3*, *NP 11-6*, *NP 17-7*) in comparison to control plant (*lane C*), **b** Ethidium bromide stained total RNA, **c** Western blot showing protein extract (50 μg) from control plant (*lane C*) and T_1 *transgenic lines* of japonica (*lanes NP 1-2*, *NP 4-3*, *NP 11-6* and *NP 17-7*)

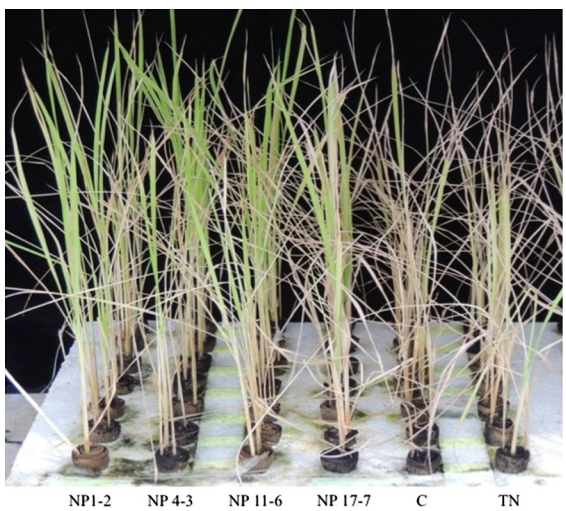

Fig. 4 Screening of T_1 transgenic japonica lines by in planta brown hopper assay. [T_1 progeny of T_0 *transgenic lines*—NP 1, NP 4, NP 11 & NP 17; *control lines* NP (Nipponbare) & TN (Taichung Native 1)]

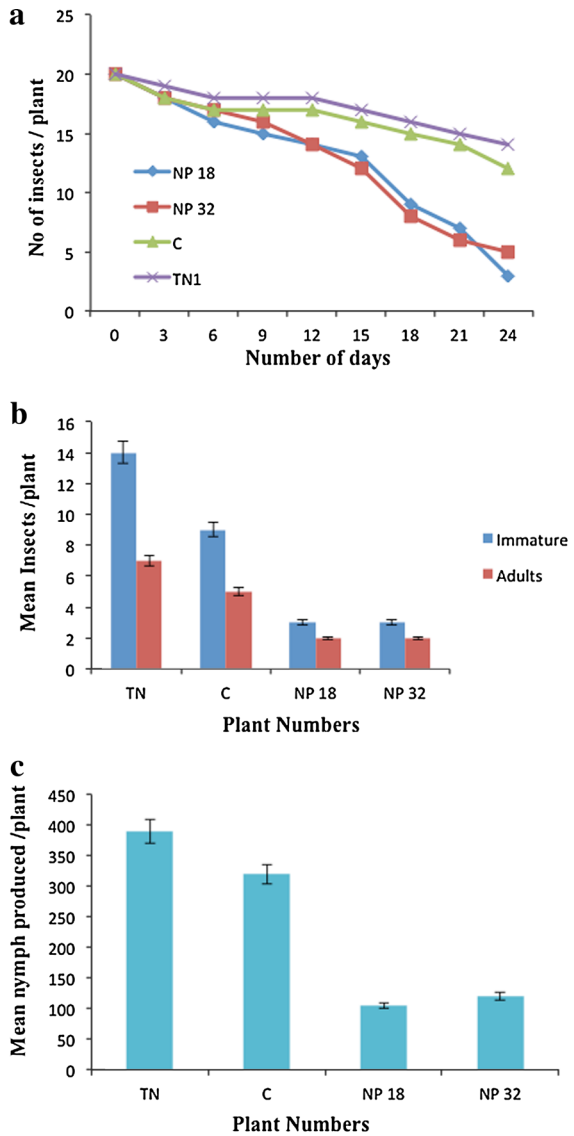

Fig. 5 Effect of ASAL expression on **a** survival, **b** development and **c** fecundity of brown hoppers. Total number of nymphs produced by a pair of brown hopper adult insects feeding on control and transgenic plants were counted and were plotted on the graph. [Bioassays were carried out on 20 plants sampled from each of the transgenic lines and control. Differences between control and transgenic plants were significant at $p < 0.005$ (ANOVA). *Bars* indicate mean ± SE; NP 18 (progeny of T_1-NP 1-2) and NP 32 (progeny of T_1-NP 11-6) are T_2 generation transgenic plants and *NP* & *TN* stand for Nipponbare and Taichung Native 1 control plants]

survival of brown hoppers was significantly reduced to 3 ± 1.1 (NP 18), 5 ± 1.6 (NP 32) insects/plant on transgenic plants in comparison to 12.4 ± 1.3 (Nipponbare), and 14.4 ± 1.6 (TN1) insects/plant on control plants. First instar nymphs of brown hopper were released on ASAL T_2 transgenic plants NP 18 (from T_1-NP 1-2) and NP 32 (from T_1-NP 11-6) to monitor the effect of ASAL on their growth and development in comparison to control plants. Insects fed on transgenic plants revealed significant delay in acquiring adulthood (i.e., 9–11 days lag) in comparison to the insects fed on control plants. Among brown hopper survivors on different transgenic lines only

11–28 % reached the adult stage in comparison to control plants that showed 74–90 % surviving adult insects (Fig. 5b). Effect of ASAL on the fecundity of brown hoppers was assessed by estimating the total number of nymphs produced by the insects fed on T_2 transgenic rice plants. The mean number of brown hopper nymphs/plant were reduced on T_2 transgenic plants i.e., 105 ± 4 (NP 18; progeny of T_1-NP 1-2), 120 ± 3 (NP 32; progeny of T_1-NP 11-6), in comparison to the number of nymphs/plant recorded on control plants i.e., 320 ± 5 (Nipponbare) and 390 ± 16 (TN1) (Fig. 5c).

Discussion

This study deals with the phloem-specific expression of *ASAL* in brown hopper-susceptible, high-yielding japonica (Nipponbare) rice cultivar for enhancing its resistance to brown hoppers. The deduced amino acid sequence of ASAL protein has maximum similarity (~98 %) with that of previously reported garlic lectin protein (Saha et al. 2006). Employing the protocols optimized in our laboratory, the co-integrated binary vector (pMDC99: RSs1: ASAL) carrying hygromycin as selective agent has been used to transform japonica rice. PCR and Southern analyses of hygromycin tolerant plants confirm the integration of *HPT* and *ASAL* genes into the japonica rice genome. Digested genomic DNA on probing with *ASAL* revealed a specific hybridizable band of ~4 kb in selected transgenic lines (Fig. 2a) thereby suggesting the independent nature of transgene integration in primary transformants.

These observations further suggest the integration of T-DNA in their genome as a single copy without any rearrangement. It has been established that multiple copies of transgene(s) often result in co-suppression and gene silencing (Shou et al. 2004). Differential transcript expression of *ASAL* gene in the transgenic plants is evidenced by varied intensity of the hybridizable band of approx. 1 kb (Fig. 3a). Western analysis of transgenic plants confirmed the stable expression of ASAL at the protein level (Fig. 3c). Marked variation observed in the amount of ASAL (0.63–0.95 %) in different transformants by ELISA analysis suggests random transgene integration at different transcriptionally active sites in their genomes. The amount of ASAL protein expression in different transgenic plants is distinctly higher in comparison to the lectin expression levels in previous studies (Saha et al. 2006; Nagadhara et al. 2003). To establish the definite transgenic nature of primary transformants, the inheritance pattern of transgenes was analyzed in the T_1 generation. Screening on hygromycin media and Southern analysis indicated that *HPT* and *ASAL* are transmitted in a Mendelian fashion. Segregation analyses of transgenes in T_1 progenies revealed a monogenic ratio of 3 (resistant): 1 (susceptible) plant(s) for both hygromycin tolerance and insect resistance. The results affirm stable integration of transgenes into the transgenic rice genomes (Supplementary Fig. 1 and Supplementary Table 1). The co-segregation of transgenes further confirms that both *HPT* and *ASAL* are integrated and manifest as a single locus. Insect bioassays "*in planta*" indicate that expression of ASAL in transgenic rice lines imparts substantial resistance against brown hopper insects, evident by decreased insect feeding and declined insect survival, thereby minimizing the hopper burn damage. Further, strong entomotoxic effects of ASAL transgenic plants against major sap-sucking pests has been demonstrated employing standard screening techniques that simulate field conditions. Progenies of two independent homozygous T_2 transgenic lines exhibited high-level resistance (1–2 score on a 0–9 scale) testifying that ASAL confers ample protection against sap-sucking insects. The 1st instar brown hopper nymphs, when fed on selected T_2 *ASAL* transgenic lines (viz., NP 18, NP 32 expressing 0.63, 0.95 % ASAL, respectively) disclosed ~50 mortality within 10–12 days of infestation (Fig. 5a). After 30 days of infestation, very few insects (2–4 per plant) could survive on transgenic plants (Fig. 5a) exhibiting delayed moulting and prolonged life cycle (~10 days) in comparison to those present on susceptible control plants. The survival of brown hopper was reduced by~ 74 % on Nipponbare transgenic lines in comparison to control plants (Fig. 5a).

Our results clearly indicate that the ASAL-expressing transgenic rice plants exhibit a higher level of resistance against brown hopper pests in comparison to GNA- (Nagadhara et al. 2003) and ASAL- (Saha et al. 2006) expressing rice lines where overexpression of ASAL and GNA in rice reduced the survival of brown hopper by 36 % (Saha et al. 2006) and 32–59 %, respectively (Nagadhara et al. 2003).

Similarly, the GLH survival was decreased by 49 to 53 % on GNA transgenic plants (Nagadhara et al. 2003) and by 32 % on ASAL-expressing plants (Saha et al. 2006). Furthermore, fecundity assays conducted on *ASAL* rice lines revealed significant decline in the nymph production of brown hopper insects by ~68 % (Fig. 5c) indicating marked decreases in the fecundity of brown hopper insects suggesting high entomotoxic effects of ASAL on these insects. A direct correlation thus occurs between the amount of garlic lectin in transgenic plants and its entomotoxic effects on sap-sucking insects. Earlier, reports showed that brown hopper nymph production was reduced by 59 %, when fed on ASAL-expressing transgenic rice (Saha et al. 2006). An overview of insect bioassays strongly establishes that ASAL is more toxic to brown hopper insects compared to GNA, under similar bioassay conditions.

The variable entomotoxic effects of GNA and ASAL proteins on sap-sucking pests may be attributed to their differential binding affinities to receptor proteins on gut epithelial cells of the insects. As it is imperative to identify eco-friendly and potent insecticidal proteins, the *ASAL* may be preferred for genetic engineering of diverse crops against sucking pests. Overall results of insect bioassays '*in planta*' establish that *ASAL* transgenic rice plants express functionally active ASAL protein, which is comparable to the effect of native garlic lectin (s) used in artificial diets against sucking insects (Nagadhara et al. 2003, 2004). Although the precise mechanism underlying the lectin toxicity to insects is unclear, it may involve binding of lectins to the receptors lining the gut epithelial cells of various insects. Immuno-histochemical studies of a wide range of mannose or mannose/glucose specific lectins such as GNA, Con A and PSA suggest their binding to the mid-gut epithelial cells of insects thereby contributing to the insecticidal effect. Moreover, the bound lectins might inhibit the absorption of nutrients or disrupt the mid-gut cells through endocytosis of lectin and other toxic metabolites. The toxicity of mannose binding lectins towards sucking insects is not clear, but it is known to bind to the mannose moiety of brush border membrane vesicle (BBMV) receptors of gut epithelial cells, thereby causing disruption of cell function and mortality (Fitches et al. 2001). Ligand blot analysis of the mustard aphid (BBMV) demonstrates that ASAL protein binds to symbionin (Sym L) receptor involved in the transmission of viruses by sucking pests (Banerjee et al. 2004).

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