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Stability of sheath blight resistance in transgenic ASD16 rice lines expressing a rice *chi11* gene encoding chitinase

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Abstract

Development of transgenic plants by introducing defense genes is one of the strategies to engineer disease resistance. Transgenic ASD16 rice plants harbouring rice chitinase *chi11* gene, belonging to a PR-3 group of defense gene conferring sheath blight (*Rhizoctonia solani* Kuhn) resistance, were used in this study. Three T_2 homozygous lines (ASD16-4-1-1, 5-1-1, and 6-1-1) were identified from seven putative (T_0) transgenic lines expressing *chi11* using Western blotting analysis. The inheritance of sheath blight resistance in those lines was studied over generations. The stability of *chi11* expression up to T_4 generation in all the three homozygous lines was proved by Western blot and the stability of sheath blight resistance in the homozygous lines was proved up to T_4 generation using detached leaf and intact leaf sheath assays. Among the three homozygous lines tested, ASD16-4-1-1 showed consistent results in all the generations and gave a better protection against the sheath blight pathogen than the other two lines.

Additional key words: gene expression stability, *Oryza sativa*, *Rhizoctonia solani*, Western blot.

Introduction

Sheath blight caused by *Rhizoctonia solani* Kuhn is an important disease of rice worldwide (Dasgupta 1992) and a yield loss ranges from 8 to 50 % depending on severity of the disease, the stage at which the crop is infected, and environmental conditions (Savary *et al.* 2000, Singh *et al.* 2004). Although the extensive use of chemicals remains the main strategy of disease control, management of sheath blight could also be achieved through transgenic approaches (Lin *et al.* 1995, Datta *et al.* 2000, 2001, Kim *et al.* 2003).

Chitinase is PR-3 group of pathogenesis related (PR) proteins that hydrolyse the β-1,4 linkage of the *N*-acetylglucosamine residue of chitin, a structural polysaccharide of the cell wall of many fungi. Chitinases purified from plants, microbes, and animals show a strong antifungal activity *in vitro* (Neuhaus 1999). In addition to this direct action, the released oligomers of *N*-acetylglucosamine function as elicitors to further amplify the defense response in cells surrounding the site of infection (Ren and West 1992). Chitinase preparation from rice plants effectively inhibits mycelial growth of the rice sheath blight pathogen.

Lin *et al.* (1995) introduced a rice gene encoding chitinase into *indica* rice cv. Chinsurah Boro II and produced transgenic rice lines with an enhanced resistance to *R. solani*. Datta *et al*. (2000) subsequently transformed rice cvs. Basmati122, Tulsi, and Vaidehi with a class-I chitinase gene *chi11* by an *Agrobacterium*-mediated transformation system. Sheath blight bioassay results show that transgenic plants expressing *chi11* restrict growth of the sheath blight pathogen, and lesion areas are smaller compared to non-transgenic control plants. Datta *et al*. (2001) introduced a rice chitinase gene (*RC7*), isolated from *R. solani* infected rice plants, into cvs. IR72, IR64, IR68899B, and Chinsurah Boro II by biolistic and polyethylene glycol-mediated transformation systems and studied inheritance up to T_2 generation. The transgenic plants show different levels of resistance upon

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Abbreviations: HAI - hours after infection; HRLH - highest relative lesion height; PR - pathogenesis related.
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inoculation with the sheath blight pathogen. Co-expression of a rice basic chitinase gene and a ribosome-inactivating protein in rice causes a significant reduction in sheath blight development (Kim *et al.* 2003). Kumar *et al*. (2003) used *Agrobacterium*-mediated transformation for introduction of *chi11* into cv. Pusa Basmati 1 (PB1), which significantly increased its resistance against the sheath blight pathogen in detached leaf and intact leaf sheath assays. Sridevi *et al.* (2003) transformed rice cv. PB1 with *chi11* by employing an *Agrobacterium*-mediated transformation system. They performed Northern and Western blotting analyses in T_1 plants and revealed a constitutive expression of chitinase at a high amount. Bioassay of T_1 plants indicated a higher resistance to *R. solani* in comparison to control plants. More resistance to *R. solani* was noticed in a homozygous transgenic line.

Yuan *et al.* (2004) reported that transgenic rice Zhongda 2, which was genetically modified from line Zhuxian B with a rice chitinase gene (*RC24*) through sexual crossing, had a high resistance to sheath blight in laboratory and field experiments. In our earlier work, we co-transformed cvs. ADT38 and IR50 with *tlp* and *chi11*, encoding 35 kDa rice chitinase, through biolistic-mediated co-transformation (Kalpana *et al.* 2006). The co-transformants expressing both *tlp* and *chi11* show an elevated resistance against *R. solani* and *Sarocladium oryzae* than plants expressing either *tlp* or *chi11*. In another experiment, we co-introduced *chi11* gene and/or *tlp* gene (*tlp-D34*) into cvs. PB1 and ADT38 using a biolistic method. The co-transformants exhibited an enhanced resistance against *R. solani* (Maruthasalam *et al.* 2007). Transgenic rice plants of local cultivars developed through *Agrobacterium*-mediated transformation expressing rice chitinase gene *RC7* exhibit an enhanced resistance (up to 33.3 %) to *R. solani* under glasshouse conditions (Nandakumar *et al*. 2007).

Sridevi *et al.* (2008) used *Agrobacterium*-mediated transformation for simultaneous introduction of c*hi11* gene and tobacco *β-1,3-glucanase* gene into cv. PB1. Three (CG20, CG27, and CG53) homozygous T_2 transgenic plants co-expressing c*hi11* and *β-1,3-glucanase* genes upon challenge inoculation with *R. solani* exhibited a 60 % reduction in sheath blight disease index in the first week compare to non-transgenic control plants. Disease index in control plants rapidly increases from 61.8 to 90.6 in 1- to 3-week period, whereas disease index remains in the range of 26.8 - 34.2 in T_3 homozygous transgenic plants during the same period (Sridevi *et al.* 2008). Shrestha *et al.* (2008) reported that the percentage of relative lesion height and the number of infection cushions are negatively correlated with chitinase activity in rice plants.

Shah *et al.* (2009) transformed cv. PB1 with an endochitinase gene (*cht42*) from a fungus *Trichoderma virens* through *Agrobacterium*-mediated transformation. Infection assay performed on homozygous T_2 plants with *R. solani* shows up to 62 % reduction in sheath blight disease index. Li *et al.* (2009) observed that transgenic rice lines and their progenies over-expressing *Momordica charantia* class I chitinase gene (*McCHIT1*) show an enhanced resistance to *R. solani* and *Magnaporthe grisea*. Recently, Shah *et al.* (2013) transformed cv. White Ponni with *tlp-D34* gene alone or in combination with *chi11* through *Agrobacterium*-mediated transformation. Co-expression of *tlp-D34* and *chi11* genes results in a much higher sheath blight disease resistance. Homozygous T_2 plants harbouring both genes exhibit the lowest disease index of 39 % against 100 % in control plants.

In the present study, the stability of sheath blight resistance was studied in three homozygous lines (ASD16) expressing *chi11* over T_2, T_3, and T_4 generations through detached leaf and intact leaf sheath assays standardised in our laboratory (Kumar *et al*. 2003). Further, the stability of *chi11* expression up to T_4 generation in homozygous ASD16 lines was analysed by Western blotting.

Materials and methods

The plant transformation vector pMKU-RF2 harbouring a 3.2 kbp chitinase gene expression cassette containing a 1.1 kbp rice *chi11* gene (a PR-3 group of defense gene conferring sheath blight resistance) under control of a ubiquitin promoter and an NOS polyA terminator was used in the transformation experiments (Kumar *et al*. 2003). Seven putative (T_0) transgenic ASD16 rice lines (ASD16-1 to 7) transformed with pMKU-RF2 earlier in our laboratory (Kalpana *et al*. 2006) were used in the present study. From the putatively transformed lines, T_1 seeds were collected and forwarded to T_2 generation. The expression of *chi11* in the T_2 progeny plants was ascertained through Western blotting analysis. The progenies of the selected *chi11* positive lines were analysed for homozygosity for chitinase expression in the subsequent (T_3 and T_4) generations.

For Western blotting analysis, leaves (250 mg) from transgenic and non-transgenic ASD16 plants were ground with 1 cm^3 of ice-cold phosphate-buffered saline (KH_2PO_4 0.2 g, Na_2HPO_4 1.15 g, KCl 0.2 g, NaCl 8 g, distilled water 1000 cm^3, pH 7.0). The extracts were centrifuged at 15 000 *g* and 4 ºC for 12 min. The aqueous phase was collected and proteins were resolved by sodium dodecyl sulphate polyacrylamide (12 %, m/v) gel electrophoresis (*Mini-PROTEAN® II* cell, *Bio-Rad*, Hercules, USA) following a standard procedure (Laemmli 1970). Western blotting analysis was carried out following the method described by Gallagher *et al.* (1995). Fractionated proteins were transferred onto a nitrocellulose membrane (*Protran BAS 5 Cellulocenitrat*, *Schleicher and Schuell*, Dassel, Germany) using a *Trans-Blot® SD* semi-dry transfer cell (*Bio-Rad*). The

membranes were then probed with an *anti*barley chitinase antibody (courtesy Dr. S. Muthukrishnan, the Kansas State University, USA). The polypeptides recognized by specific antibodies were detected using a goat *anti*rabbit IgG-alkaline phosphatase conjugate by incubating the membrane in the dark with a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium liquid substrate system (*Sigma-Aldrich*, St. Louis, USA). This study was performed to confirm the stability of *chi11* expression in T_2, T_3, and T_4 progenies of the transgenic ASD16 lines. Three transgenic ASD16 lines (ASD16-4-1-1, ASD16-5-1-1, and ASD16-6-1-1), identified to be homozygous for *chi11* expression, were used for further study.

These homozygous lines were evaluated for sheath blight resistance along with non-transgenic ASD16 control plants. Two different sheath blight assay methods, one using detached leaves and another with intact leaf sheaths, were employed as described by Kumar *et al.* (2003). All the inoculations were carried out with 5 mm mycelial discs obtained from 3-d-old *Rs*7 (a virulent isolate of *R. solani*; Krishnamurthy *et al.* 1999) maintained on potato dextrose agar (250.0 g dm^{-3} potato, 20.0 g dm^{-3} dextrose, and 20.0 g dm^{-3} agar, pH 7.0). In bioassay using detached leaves, observations were made at 24-h intervals, and infection cushions were counted under a stereomicroscope 72 h after inoculation (HAI). In bioassay using intact leaf sheaths, observations were made up to 7 d at 24-h intervals. At 168 HAI, the highest relative lesion height percentage, HRLH [%] = (length of the highest lesion/plant height) × 100, and the total lesion spread were estimated. These experiments were carried out simultaneously in the T_2, T_3, and T_4 progeny plants.

All the bioassay experiments were carried out in a completely randomized design with adequate replications. Duncan's multiple range test (DMRT; Gomez and Gomez 1984) was used to compare treatment means using the software *IRRISTAT v. 3.1* developed by the Biometrices Unit, the International Rice Research Institute, Los Banos, Manila, The Philippines.

Results

The *anti*barley chitinase antiserum detected the expression of a 35 kDa polypeptide in chitinase positive lines of T_1 generation. The selected positive lines from seven T_1 lines were forwarded to the next generation (Table 1). In T_2 generation, all the 19 progeny plants of the ASD16-4-1-1, ASD16-5-1-1, and ASD16-6-1-1 transgenic lines showed a stable expression of the 35 kDa chitinase polypeptide confirming their homozygosity for *chi11* expression (Table 1).

The stability of *chi11* expression in those lines was studied over generations T_2 to T_4 through Western blotting analysis. In all the generations, an immunologically-related polypeptide with an apparent molecular mass of 35 kDa was found indicating the stability of chitinase expression up to T_4 generation (Fig. 1). Apart from the expected 35 kDa polypeptide, a 30 kDa polypeptide signal was also found in most of the transgenic lines with varying intensities (Fig. 1). However, no such bands were apparent in the non-transgenic control plants.

Progenies (T_2, T_3, and T_4) of the three homozygous lines were evaluated for sheath blight resistance through detached leaf and intact leaf sheath assays using a virulent *Rs*7 isolate. Formation of infection cushions away from the site of inoculation was induced by *Rs*7 isolate in both the transgenic plants and the non-transgenic plants at 48 HAI. The detached leaf assay exhibited a reduction in number of infection cushions on leaves of the transgenic plants coupled with browning around the infection cushions, and the leaves remained green, whereas the non-transgenic plants showed an increased number of infection cushions followed by yellowing and drying the entire leaf blade at 72 HAI

Table 1. Western blotting analysis in T_1 and T_2 progenies of ASD16 lines carrying a *chi11* gene.

Lines	Generations	Number of plants analysed	Number of positive plants
ASD16-1	T_1	12	10
ASD16-2	T_1	4	3
ASD16-3	T_1	14	6
ASD16-4	T_1	7	7
ASD16-5	T_1	8	8
ASD16-6	T_1	10	10
ASD16-7	T_1	5	4
ASD16-4-1	T_2	19	19
ASD16-5-1	T_2	19	19
ASD16-6-1	T_2	19	19

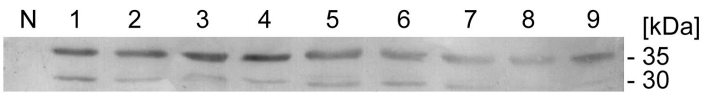

Fig. 1. Western blot analysis of chitinase expression in T_2, T_3 and T_4 progenies of transgenic homozygous lines ASD16-4-1-1, ASD16-5-1-1, and ASD16-6-1-1. A 35 kDa polypeptide is possibly product of the introduced (*chi11*) gene, and a 30 kDa band is probably protein product released from the 35 kDa polypeptide during boiling (Datta *et al*. 2000). *Lane N* - non-transgenic control, *lane 1* - ASD16-4-1-1 (T_2), *lane 2* - ASD16-4-1-1 (T_3), *lane 3* - ASD16-4-1-1 (T_4), *lane 4* - ASD16-5-1-1 (T_2), *lane 5* - ASD16-5-1-1 (T_3), *lane 6* - ASD16-5-1-1 (T_4), *lane 7* - ASD16-6-1-1 (T_2), *lane 8* - ASD16-6-1-1 (T_3), and *lane 9* - ASD16-6-1-1 (T_4).

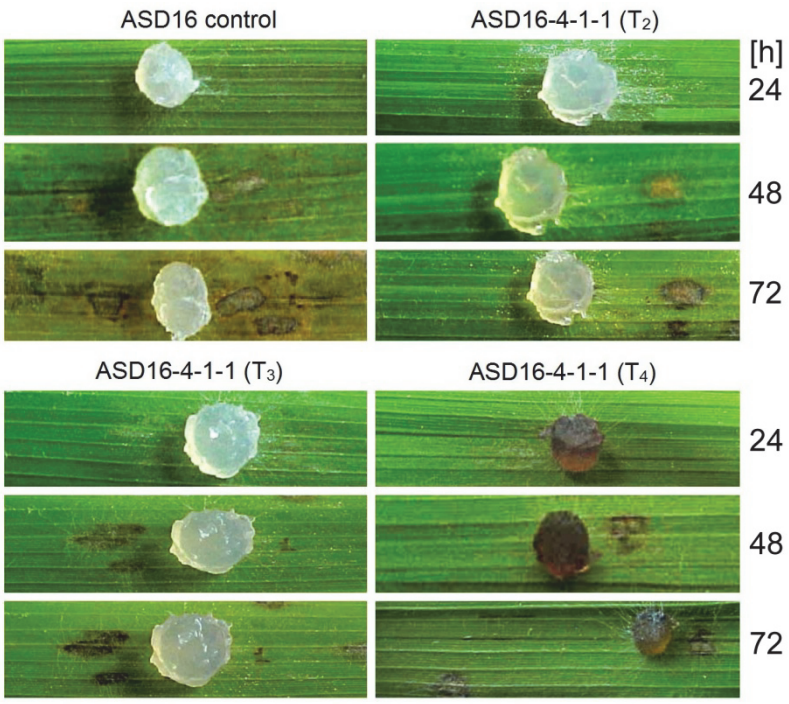

Fig. 2. Assessment of sheath blight resistance by detached leaf assay in homozygous transgenic line ASD16-4-1-1 expressing *chi11*.

Table 2. Assessment of sheath blight resistance (numbers of infection cushions on detached leaves). Means of three replications. In a column, the means followed by different letters are significantly different at the 5 % level by Duncan's multiple range test.

Lines	T_2	T_3	T_4
ASD16-4-1-1	13.2^a	12.7^a	10.8^a
ASD16-5-1-1	15.9^b	17.2^c	14.3^b
ASD16-6-1-1	18.1^c	14.0^b	16.1^c
ASD16 control	50.5^d		

(Fig. 2, Table 2). The frequency of formation of infection cushions was as high as 50.5 in the ASD16 control, whereas it was only 10.8, 14.3, and 16.1 in T_4 progenies of lines ASD16-4-1-1, ASD16-5-1-1, and ASD16-6-1-1 expressing *chi11* (Table 2). A similar frequency of infection cushion formation was observed in T_2 and T_3 progenies. In general, the number of infection cushions formed was significantly lower (23 - 40 %) in T_2, T_3, and T_4 progenies of ASD16-4 line when compared to the progenies of ASD16-5 and ASD16-6 lines (Table 2).

The total lesion spread and HRLH [%] are the characteristics used to study a relative resistance/ susceptibility of the transgenic and non-transgenic lines to *R. solani*. Intact leaf sheath assay showed an early appearance (within 48 HAI) of a symptom on leaf sheaths of the non-transformed plants, whereas the blighting was delayed even up to 120 HAI in the transgenic T_4 ASD16 lines constitutively expressing the rice chitinase (Fig. 3). Moreover, lesion spread was very much reduced on sheaths of all the transgenic lines because of extensive browning around the lesions, whereas light grayish lesions were formed on sheaths of the non-transformed plants followed by pathogen spread over the entire leaf sheaths leading to complete drying of infected leaf sheath within 168 HAI (Fig. 3).

The non-transgenic plants recorded a HRLH of 8.15 % at 168 HAI, whereas it was restricted to 2.12, 4.0,

Table 3. Assessment of sheath blight resistance by intact leaf sheath assay. Means of three replications. In a column, the means followed by different letters are significantly different at the 5 % level by Duncan's multiple range test. HRLH - highest relative lesion height.

Line	HRLH [%]			Total lesion spread [cm]		
	T_2	T_3	T_4	T_2	T_3	T_4
ASD16-4-1-1	2.28^a	2.54^a	2.12^a	3.44^a	3.41^a	3.14^a
ASD16-5-1-1	3.97^b	4.05^b	4.00^b	5.10^b	4.44^b	4.31^b
ASD16-6-1-1	4.60^c	4.29^c	4.24^c	5.73^c	5.51^c	5.03^c
ASD16 control	8.15^d			10.08^d		

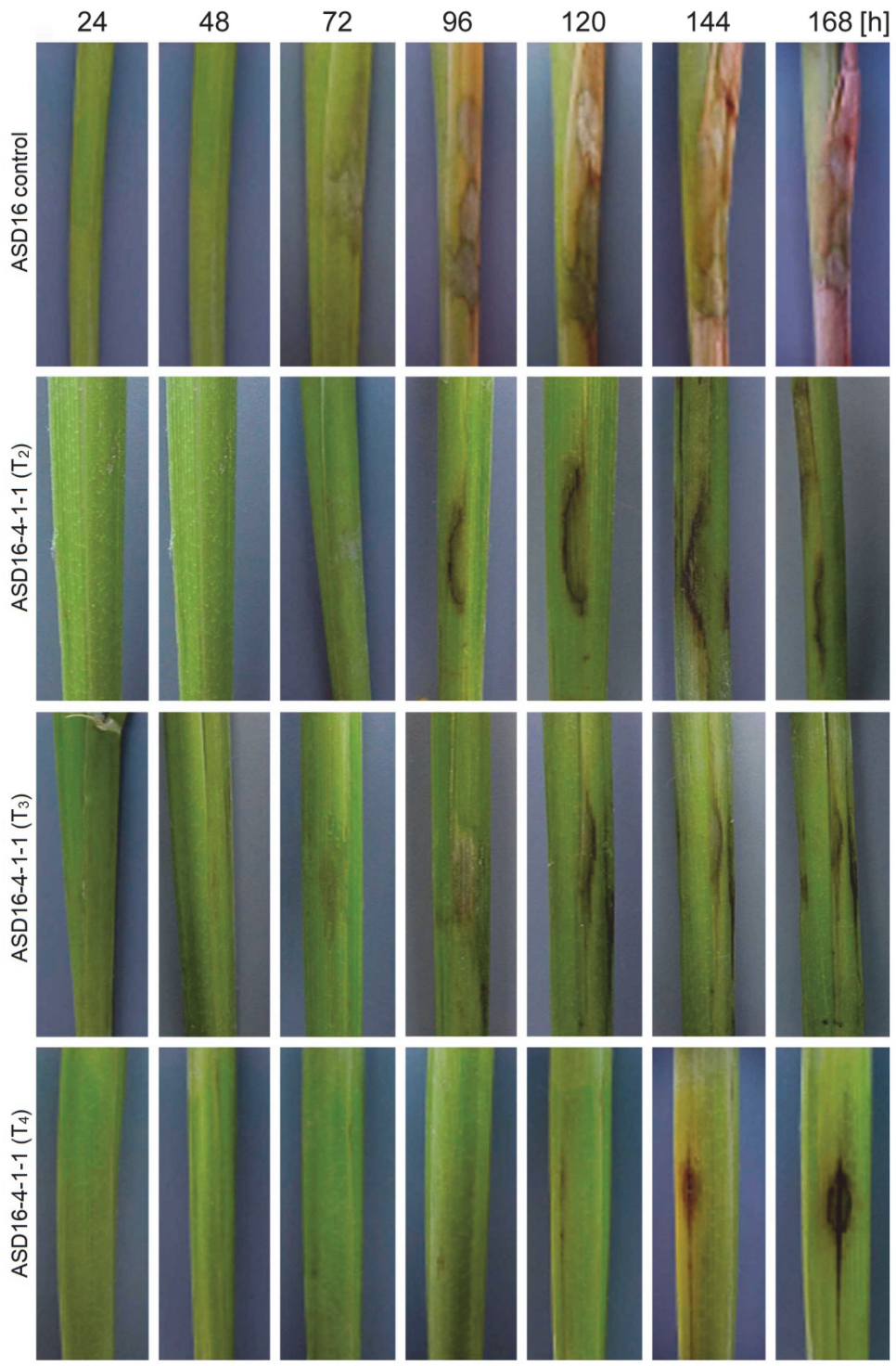

Fig. 3. Assessment of sheath blight resistance by intact leaf sheath assay in homozygous transgenic line ASD16-4-1-1 expressing *chi11*.

and 4.24 % in transgenic T_4 lines ASD16-4-1-1, ASD16-5-1-1, and ASD16-6-1-1, respectively (Table 3). Similar results were also obtained in T_2 and T_3 progenies of all the three lines (Table 3). The HRLH was very much reduced in T_2, T_3, and T_4 progenies of ASD16-4 with 2.28, 2.54, and 2.12 %, respectively (Table 3).

The total lesion spread on leaf sheaths of transgenic T_4 lines ASD16-4-1-1, ASD16-5-1-1, and ASD16-6-1-1 (168 HAI) was limited to 3.14, 4.31, and 5.03 cm, respectively, as compared to 10.08 cm in non-transgenic

ASD16 control (Table 3). Similarly, T_2 and T_3 progenies of all the three lines showed a significant reduction in total lesion spread compared to the control (Table 3). Among the three homozygous ASD16 lines tested, ASD16-4 was found to be the best performing line in all the three generations tested and conferred an enhanced protection against *R. solani* than other two lines.

Discussion

The role of chitinase in plant defense against fungal attack has been well documented (Adams 2004, Tohidfar *et al.* 2009). The chitinase gene was successfully transformed and expressed in tobacco (Brogue *et al.* 1991), rice (Lin *et al.* 1995), strawberry (Asao *et al.* 1997), pigeon pea (Kumar *et al.* 2004), cotton (Tohidfar *et al.* 2005, 2009), taro (He *et al.* 2008), and grapevine (Nirala *et al.* 2010). Several reports have indicated the potential of chitinase gene in enhancing the resistance against sheath blight and/or sheath rot pathogens in rice (Lin *et al.* 1995, Nishizawa *et al.* 1999, Datta *et al.* 2000, 2001, Itoh *et al.* 2003, Kim *et al.* 2003, Kumar *et al.* 2003, Sridevi *et al.* 2003, 2008, Kalpana *et al.* 2006, Maruthasalam *et al.* 2007, Nandakumar *et al.* 2007).

In this work, the *anti*barley chitinase antiserum detected the expression of a 35 kDa polypeptide in the plants of T_2, T_3, and T_4 progenies of three homozygous lines (Table 1). In addition, another band of 30 kDa was found in all the transgenic plants (Fig. 1) and was absent in the non-transgenic control. Lin *et al*. (1995) and Datta *et al*. (2000) reported that the second chitinase band of about 30 kDa may be a truncated form of original 35 kDa chitinase released by proteolytic processing during boiling the crude protein extract. The banding pattern observed in this study confirms our earlier reports (Kumar *et al.* 2003, Kalpana *et al*. 2006, Maruthasalam *et al.* 2007). Datta *et al.* (2001) found the expression of 35 kDa chitinase (*RC7*) in different generations of transgenic lines of *indica* rice cvs. IR64 and IR72 developed through a biolistic method. Nandakumar *et al.* (2007), Sridevi *et al.* (2008), and Shah *et al.* (2013) detected the expression of 35 kDa rice chitinase by a polyclonal barley chitinase antibody in different tissues of transgenic plants by Western blot.

The stability of sheath blight resistance over generations (up to T_4) in the homozygous lines expressing *chi11* was proved by both detached leaf assay and intact leaf sheath assay. The present study using detached leaves revealed that the leaves of the homozygous transgenic plants of all three generations exhibited characteristic browning around the lesions. This could be attributed to an effective inhibition of pathogenic invasion in leaves expressing chitinase (Kumar *et al*. 2003, Maruthasalam *et al*. 2007). Rapid yellowing and drying observed in leaves of the non-transgenic control was absent in the transgenic plants. Moreover, the number of infection cushions formed on leaves was significantly lesser in the transgenic lines expressing *chi11* and their progenies as compared to the non-transgenic control.

In intact leaf sheath assay, precocious browning at the site of inoculation was observed in the transgenic plants of all three generations and such browning might play a major role in arresting the pathogen spread. In the non-transgenic control plants, browning was absent and drying leaf sheaths was noticed, whereas leaf sheaths of the transgenic plants expressing *chi11* remained green despite sheath blight infection. This clearly demonstrates that pathogen spread was very much restricted in the transgenic lines possibly due to extensive browning at the site of inoculation. Extensive browning could be a defensive strategy by the host to restrict pathogen invasion. Previous reports suggested that browning may be due to the production of oxidized phenolics around the infection court (Groth and Nowick, 1992), hypersensitive reaction (Yamamoto *et al*. 2000, Maruthasalam *et al.* 2007), and rapid cell death mediated by the expression of defense genes upon pathogen invasion (Heath 2000). Similar results were obtained for transgenic rice plants expressing *chi11* in detached leaf assay and intact leaf sheath assay by our group earlier (Kumar *et al.* 2003 Maruthasalam *et al.* 2007). They have also reported a delayed symptom development and profuse browning in transgenic plants.

Datta *et al.* (2001) observed that the number of lesions in transgenic plants expressing rice chitinase (RC7) was lesser and their sizes smaller than those in control plants. Average sheath infection rates on the control plants of IR72 and IR64 were 27.7 and 31.3 %, respectively, whereas they were limited to 12.6 and 8.5 % in two transgenic homozygous lines of IR72 and 11.1 % in IR64. Yuan *et al.* (2004) reported a disease index of 18.1 to 26.1 in transgenic Zhongda 2 expressing a rice chintinase (*RC24*) gene, whereas it ranges from 40.6 to 59.4 and 32.5 to 35.1 in non-transgenic Zhuxian B and Zhan A plants, respectively. Disease index of Zhongda2 was 18.1 to 26.1, whereas that of Zhuxian B and Zhan A (non-transgenic lines) was from 40.6 to 59.4 and 32.5 to 35.1, respectively. A characteristic brown necrotic symptom appeared in both the transgenic lines and the non-transgenic lines in two days after inoculation, but spot length in Zhongda 2 was smaller than that of Zhuxian B. Ten days post inoculation, the average length of a necrotic spot in Zhongda 2 was limited to 6.1 cm and also spot size increased very slowly, whereas spot size of Zhuxian B grew much faster.

Nandakumar *et al.* (2007) reported that transgenic rice lines expressing enhanced resistance to sheath blight produce fewer lesions registering disease grades of zero to three, whereas it is four to five in non-transgenic lines inducing larger lesions. Sridevi *et al.* (2008) recorded a 60 % reduction of sheath blight disease index in

homozygous T_2 and T_3 plants of *indica* rice cv. PB1. Sheath blight resistance in CG20, CG27, and CG53 homozygous lines is inherited from T_2 to T_3 generation and persists up to three weeks after inoculation with *R. solani*. In the present study, we also observed the inheritance of sheath blight resistance in three generations of homozygous ASD16 lines (T_2 to T_4) expressing *chi11*. Shrestha *et al.* (2008) has found a negative correlation between the percentage of relative lesion height and chitinase activity in different rice cultivars. The chitinase activity and the number of infection cushions formed by *R. solani* are also negatively correlated indicating that the sheath blight resistance is correlated with the increase in chitinase activity.

Li *et al.* (2009) transformed *indica* rice cv. JinHui35 with bitter melon class I chitinase gene *McCHIT1* using *Agrobacterium*-mediated transformation. They performed sheath blight and blast bioassays with six transgenic lines from T_0 to T_2 generation. Bioassay of both T_1 and T_2 generations shows an enhanced disease resistance. The T_2 lines show an increased resistance to *R. solani* with a reduction of disease index ranging from 25.0 to 43.0 as compared to control plants. They also observed that both T_1 genration and T_2 generation show a higher resistance to a blast pathogen *Magnaporthe grisea* than control plants. Shah *et al.* (2009) reported that sheath blight index in homozygous T_2 transgenic plants expressing a 42 kDa endochitinase (*cht*42) gene from *Trichoderma virens* is much lower (22.2 - 39.3) in comparison with 58 in control plants. Recently, Shah *et al.* (2013) reported that co-expression of *tlp-D*34 + *chi11* genes in *indica* rice cv. White Ponni bring about a significantly greater reduction in sheath blight disease index in comparison to transgenic plants expressing the *tlp-D*34 gene alone. A T_2 homozygous line CT22 exhibits the lowest disease index of 39 % against 100 % in control. In all the published reports, the common phenomenon is that the transgenic homozygous lines showing a stable inheritance and expression of introduced defense gene(s) conferrs an appreciable resistance against fungal pathogens than the segregating progenies. Therefore, it is of a great importance that the integrated transgene is stably inherited in subsequent generations. In our study, the results of Western blot and pathogen bioassays clearly show the stability of *chi11* expression and sheath blight resistance over three generations (T_2, T_3, and T_4) of the transgenic homozygous ASD16 lines. The expression of *chi11* gene in all three generations was similar in terms of sheath blight protection conferred by them. In the present study, among three homozygous ASD16 lines tested, ASD16-4-1-1 performed a better protection against *R. solani* than the other two lines. It could be an excellent source of sheath blight resistance. Hence, this line could be well utilized in future breeding programs which will aim at developing rice cultivars with stable sheath blight resistance over generations.

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