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Expression of a rice chitinase gene enhances antifungal potential in transgenic grapevine (*Vitis vinifera* L.)

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Summary

To enhance the antifungal potential of grapevine, transgenic plants were generated by transferring rice chitinase gene under a maize-ubiquitin promoter along with its first intron into the leaf disc-induced somatic embryos *via Agrobacterium* mediated transformation. After co-cultivation for 2 days with recombinant *Agrobacterium*, somatic embryos were transferred onto WPM medium containing BAP 1.5 µM and NAA 0.1 µM supplemented with 25 mg/L hygromycin. Secondary or tertiary embryos were selected and the antibiotic resistant transgenic plantlets were analyzed. The integration and stability of the transgene were confirmed by PCR, RT-PCR, Southern blotting and by Western blot analyses. The transgenic plants exhibited higher chitinase activity than the non-transformed plants. These analyses indicated that the foreign gene was translated into the protein of expected molecular weight that showed chitinase activity. Following *in vitro* inoculation of powdery mildew (*Uncinula necator*), the transgenic plants showed delayed onset of the disease and smaller lesions. The transgenic plants were adapted to the greenhouse and did not show any phenotypic alterations.

K e y w o r d s : *Agrobacterium tumefaciens*, Somatic embryogenesis, Powdery mildew, Transformation, *Vitis vinifera*, Fungal tolerance.

A b b r e v i a t i o n s : NN: Nitsch and Nitsch, 2,4-D: 2,4-dichlorophenoxyacetic acid, IASP: indole-3-aspartic acid, ABA: Abscisic acid, IBA: indole 3-butyric acid, WPM: woody plant media, NAA: Naphthylphthalamic acid.

Introduction

Grapes are cultivated commercially worldwide in more than 60 countries in about 75,866 sq. km. area (FAO, 2005). Grapevine (*Vitis vinifera*) is economically one of the most important fruit crops and it is used in producing wine, juice, table grapes and dried fruit. In India it is grown in three distinct agro-climatic zones *i.e.* sub-tropical, hot tropical and mild tropical climatic regions. Major grape varieties produced in India are 'Thomson Seedless', 'Pusa Seedless' and 'Beauty Seedless' etc. However, *V. vinifera* is susceptible to an array of diseases. Fungal diseases, which cause extensive losses in yield and quality, have been the most serious problems for grapevine cultivation. The most important among them are powdery and downy mildew caused by *Erysiphe necator* [(Schwein.) Burr.] and *Plasmopara viticola* (deBary) respectively. Indian grapevine cultivars are mostly threatened by these two pathogens. Control is generally achieved by widespread application of fungicides. The economic cost and negative environmental impact associated with these applications have led to a recent search for alternative strategies, involving genetic manipulation of host defense mechanisms which offers a new perspective for introducing traits like disease resistance into the cultivars of *Vitis vinifera.*

Somatic embryogenesis has been the most favoured regenerative protocol for transformation of grapes (Mullins and Srinivasan 1976, Martinelli *et al*. 1994, Perl *et al*. 1996, Kikkert *et al*. 1996, Das *et al*. 2002, 2005). Other methods involve the transformation of meristematic bulk tissues derived from adventitious shoots (Mezzetti *et al*. 2002), embryogenic calli derived from leaves (Yamamoto *et al*. 2000), immature anthers and ovaries (Wang *et al*. 2005, Vidal *et al*. 2006). A recently described method involves the transformation of shoot apical meristems of *V. vinifera* 'Thompson Seedless' (Dutt *et al*. 2007).

Gene transfer to grapevine has been achieved by various methods including *Agrobacterium*-mediated transformation (Yamamoto *et al*. 2000, Das *et al*. 2002, Bornhoff *et al*. 2005, Wang *et al*. 2005, Dutt *et al*. 2007, He *et al*. 2008), particle bombardment (Vidal *et al*. 2006) and transfection or electroporation of protoplasts (Perl and Eshdat 1998). *Agrobacterium*-mediated transformation has been most widely used and is compatible with both the production of cell suspension cultures and the regeneration of transgenic plants from a variety of cultivars (Perl *et al*. 1996).

Pathogenesis-related protein encoding genes when over-expressed in crop plants have been shown to enhance resistance to many fungal diseases (Jayraj *et al*. 2004, Punja 2006). There are many reports in which rice chitinase gene has been shown to confer resistance against herbicide bialaphos in bread wheat (Chen *et al*. 1998), resistance in rice against the sheath blight (Lin *et al*. 1995), enhanced resistance in strawberry against a fungal pathogen *Sphaerotheca humuli* (Asao *et al*. 1997), enhanced resistance to taro (*Colocasia esculenta* (L.) against *Sclerotium rolfsii* (He *et al*. 2008), and resistance to *Fusarium sp.* in pigeon

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pea (Kumar *et al*. 2004). Yamamoto *et al*. (2000) also reported the rice chitinase gene (*RCC2*), classified as class I chitinase, expressed in grapevine for enhanced resistance to fungal pathogens.

In this paper, we report on the use of *Agrobacterium*-mediated transformation to express another rice chitinase gene pGL2 (*CaMV-Ubi-Chi11*) in *V. vinifera* var. 'Pusa Seedless'. The transgenic plants that exhibited higher chitinase activity than non-transgenic plants showed increased tolerance to powdery mildew.

Material and Methods

Plant material: The long term somatic embryogenic culture was developed from the vegetative tissues of leaves of *Vitis vinifera* L. 'Pusa Seedless' as described by Das *et al.* (2002, 2005), with slight modifications. Briefly, embryogenic calli were maintained on solidified (7 % agar) NN (Nitsch and Nitsch 1969) medium supplemented with 6 % sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 5 mg/L indole-3-aspartic acid (IASP), 0.2 mg/L 6-benzyladenine (BAP) and 1 mg/L abscisic acid (ABA). Proembryogenic calli were induced by transferring the calli to NN medium supplemented with the same phytohormones, but 2,4-D was substituted with 0.1 mg/L indole 3-butyric acid (IBA). The embryogenic cultures obtained from this stage were used for transformation experiment.

Transformation of grapevine: Embryogenic calli of grapevine (*Vitis vinifera* 'Pusa Seedless') were transformed as described by Das *et al*. (2002) by infection with *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary vector pGL2 (*CaMV-Ubi-Chi11*), which contains the *chitinase* coding region (GenBank Acc. No. X54367). Following three d of co-cultivation, the embryogenic calli were sub-cultured following the protocol of Perl *et al.* (1996). Subsequently, the calli were collected and transferred to WPM (Lloyd and McCown 1980) containing BAP 2.5 µM +NAA 0.1 µM with 25 mg/L hygromycin and 400 mg/L cefotaxime (0.7 % agar, pH 5.7) for selection. The cultures were maintained at 25 °C under a 16-h photoperiod. Shoot elongation, proliferation, rooting and subsequent plantlet development were induced on half concentration of WPM (BAP 0.25 µM + NAA 0.1 µM) supplemented with antibiotics. Rooted plantlets were further acclimatized in vermiculite and finally transferred into garden soil and grown in green house.

Polymerase chain reaction (PCR): To confirm the presence of the *chitinase* gene in transgenic plants, genomic DNA was isolated from 0.5 g of fresh young grape leaves described by Lodhi *et al*. (1994). For the PCR analysis, 200 ng of plant DNA or 4 ng of plasmid DNA was used per 25 µL reaction mixure. The primers were designed to amplify 350-bp fragments of rice chitinase gene at 63.6 °C

(F 5'-GCTACTGCTTCAAGGAGGAGAAACA-3'; R 5′-CTGGTTGTAGCAATCCAGGTTATCG-3') and 508-bp fragments of *hpt* gene at 52 °C (F 5'AGCTGCGC CGATGGTTTCTACAA3'; R 5'ATCGCCTCGCTCCAG TCAATG 3'). The PCR program profile for both the genes was as follows; initial denaturation at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 50 s at the annealing temperature of each gene and 1 min at 72 °C, with a final extension at 72 °C for 10 min. The amplified products were separated on 1 % agarose gel and visualized by staining with ethidium bromide.

Southern blot analyses: In order to confirm the transgene integration and to determine the number of copies of transgene *(Chi11*) integrated, Southern blotting (Southern 1975) experiments were performed. Genomic DNA (10 µg) and plasmid as positive control were digested with *Pst*I or *Sac*II (New England Biolab), and fragments were separated on 1 % agarose gels at 25 V for 16 h. The fractionated DNA was denatured with 0.5 M NaOH for 30 min, neutralized with 25 mM sodium phosphate buffer, pH 6.5 and transferred onto positively-charged nylon membranes (Hybond N+, Amersham Biosciences). The transferred DNA was fixed to the membrane by UV irradiation (12×10^4 µJ·cm^{-2}) using a UV cross-linker (UV-Stratalinker). Blots were hybridized with [α-^{32}P] dCTP-labelled PCR-derived pGL2 (*CaMV-Ubi-Chi11*) or *hpt* gene sequences as a probe using random primers labelling kit (New England Biolab) and detection was made by autoradiography carried out on Kodak X-ray film for 1 d.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis: Total RNA was prepared from leaf tissues using Trizol Reagent as per the manufacturer's instructions (Trizol Reagent, Invitrogen Life Technologies). To detect the presence of rice chitinase mRNA and grape actin transcripts in transformants, RT-PCR was carried out on 5 µl of RT product using pGL2 (*CaMV-Ubi-Chi11*) or actin gene specific primers at 52 °C (F 5'-TCTCCTTCAA-GACGGCGTTCTGGTTC-3'; R5'-CTGGTTGTAG-CAATCCAGGTTATCG-3'), (Act F5'-GGTAACATTGT-GCTCAGTGGTGG-3'; Act R 5'-AACGACCTTAATCT-TCATGCTGC-3') and cycle parameters were kept as mentioned in the earlier Section to amplify 290-bp of rice chitinase and 250-bp of grape actin genes. Untransformed plants cDNA was used as an experimental control.

Chitinase assay: The chitinase levels in transgenic grapevine were determined by colorimetric enzyme assay, and western blot analysis. Total soluble proteins were extracted from frozen leaves of the transformed and non-transformed samples. Leaves were homogenized with a pestle and mortar in liquid nitrogen and the frozen powder was suspended in 5 volumes of 0.1 M sodium citrate buffer (pH 6.0) containing 20 mM sodium ascorbate and polyclar AT. After two rounds of centrifugation at 13,000 rpm for 15 min at 4 °C, the supernatant was recovered (Yamamoto *et al*. 2000). The protein concentration in the extracts was estimated by the Bradford method (Bradford 1976). Equal amount (25 µg) of soluble proteins were loaded in 12 % SDS-gels and stained with Coomassie brilliant blue dye to ensure the equal loading of protein and proceeded for Western blotting by transferring proteins on to nitrocellulose membrane and probed with antiserum to bean chitinase using horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG as the secondary antibody (generously provided by Prof. S. Muthukrishnan, Kansas State

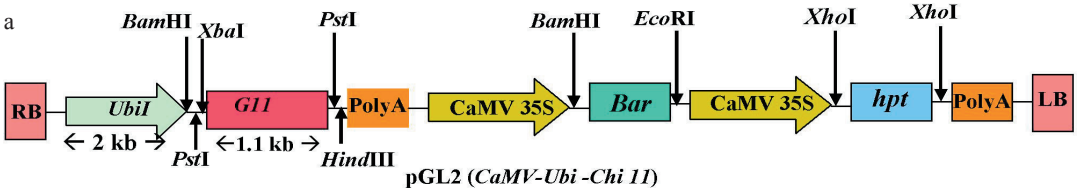
University, USA) at 1:5000 dilution. The specific position of antigen-antibody complex on the membrane was visualized on Kodak X-ray film by using super signal west pico chemiluminescent substrate (Thermo Scientific).

Quantitative assay of chitinase activity: A solublized, ethylene glycol-chitin (Sigma-Aldrich) was used as a substrate for chitinase activity assay. The colorimetric analysis of chitinase enzyme activity of PCR, Southern and RT-PCR positive transgenic plants was done following the protocol of Stephan and Wolf (1990) with slight modifications in triplicate. Aliquots of 300 µL of ethylene glycol-chitin (stock 2 mg/mL) were mixed with 100 µL of 200 mM sodium acetate buffer, pH 5.0 and 0.5 mL enzyme solution, and then incubated for 60 min. at 37 °C in circulating water bath. The reaction was terminated by addition of 100 µl HCl (1.0 N) on ice and incubated for 10 min. to facilitate precipitation of the non-degraded substrate (chitin), and then centrifuged at 14,000 g for 5 min. The resulting N-acetyl glucosamine (GlcNAc) residues were colorimetrically measured by the dinitrosalicylic acid (DNSA) method (Miller 1959). To 1 mL of the reaction mixture, 1 mL of DNSA was added and boiled for 10 min. and then 0.4 mL of potassium-sodium tartarate was added. The mixture was cooled at room temperature and OD was measured at 540 nm. As appropriate controls, the enzyme and substrate blanks were included in the experiment. One unit was defined as the amount of enzyme that produced 1 µmol of reducing sugars corresponding to *N*-acetyl-D-glucosamine in one min.

Pathogenicity test against powdery mildew: We evaluated the potential of tolerance of transgenic grapevine transformed with pGL2 (*CaMV-Ubi-Chi11*) to powdery mildew caused by *Erysiphe necator*. Newly developing secondary or tertiary leaves were used from *in vitro* grown transgenic plants. As a control, leaves from *in vitro* grown non-transformed plants were taken. *Erysiphe necator* is an obligate biotroph, so cannot be cultured on medium. The spores were collected by washing powdery mildew infected leaves from vineyards of the Indian Agricultural Research Institute, New Delhi, in distilled water containing 0.01% (v/v) Tween 20. The spore suspension (0.5 mL) was sprayed on to leaves and kept at saturated humidity at 25 °C. The degree of disease severity was scored using a visual assessment scale based on the size and intensity of powdery mildew growth. A 5-point disease rating scale based on the approximate percentage of growth of powdery mildew on the leaf surface after 15-28 d of inoculation (1 = 0 %; 2 = 1-20 %; 2 = 20-30 %; 3 = 30-40 %; 4 = 40-50 %; 5 = >50 %) (Yamamoto *et al*. 2000, Jayaraj and Punja 2007) was employed. We also recorded the number of days required for the onset and complete chlorosis in each leaf in comparison to control. Each disease value was taken on averages of three experiments and all data were analyzed for significance differences by one way analysis of variance (ANOVA) for leaf assay infection scores followed by Tukey's multiple comparison tests using the Graph Pad software (Graphpad In Stat. Software Inc. San Diego, USA).

Results

Transformation of grapevine with the rice chitinase gene: Embryogenic calli were transformed by infecting tiny globular embryos on the surface with the *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pGL2 (*CaMV-Ubi-Chi11*) (Fig. 1 a). The globular embryos were formed after transfer of proembryogenic callus to NN medium supplemented with 0.1 mg/L IBA (Fig. 1 b). Under these condi-



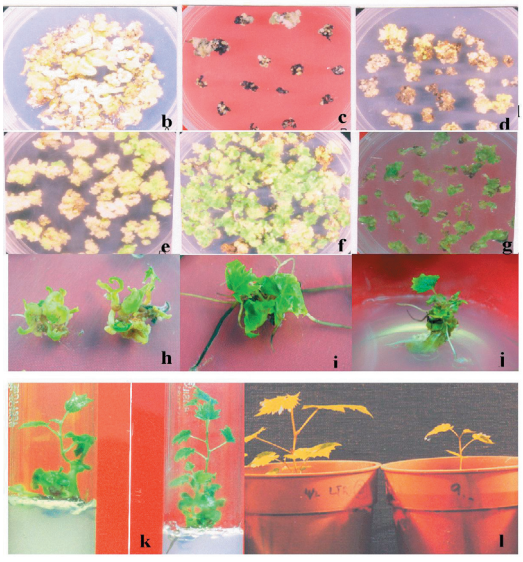


Fig. 1: **a**: A map of the T-DNA construct used for transformation of grapevine 'Pusa seedless'. Expression of gene pGL2 (*CaMV-Ubi-Chi11*) is driven by the constitutive maize ubiquitin promoter and the selectable marker *hpt* gene is under the control the constitutive 35S promoter; **b**: production of transgenic grapevine plants with the rice chitinase gene pGL2 (*CaMV-Ubi-Chi11*) using Agrobacterium-mediated transformation. Embryogenic calli of 'Pusa Seedless' obtained on NN medium containing IBA; **c**, **d**: transformed embryos selected on woody plant medium (WPM) containing 20 mg/L hygromycin after co-cultivation with *A. tumefaciens*; **e**, **f**: organization of shoots on calli of somatic embryos on the same medium containing BAP and NAA; **g**, **h**: secondary somatic embryos with its emerging plantlets on same medium; **i**, **j**, **k**: fully grown plantlets; **l**: plants in vermiculite.

tions we were able to block necrosis following the protocol of *Agrobacterium* co-cultivation with slight modification in the method of Perl *et al.* (1996).

Initially after 20 d on medium containing 10 mg/L hygromycin, a high number of germinated somatic embryos were obtained. When transferred to medium containing higher concentration of the antibiotic (15, 20 mg/L), many of the regenerants became yellowish, and were discarded after 50 d. Stable secondary and tertiary embryos were selected after additional 20 d of incubation on WPM supplemented with BAP and NAA containing 25 mg/L hygromycin and 400 mg/L cefotaxime. During *in vitro* selection, only the transformed cells maintained a high regenerative capacity, while the other parts became progressively necrotic.

About twenty putative transformed embryogenic clusters developed from initial embryogenic calli were used for co-cultivation and selection on hygromycin containing medium (Fig. 1 c, d). The shoots emerged after about 4 weeks of culture on the shoot inducing medium (Fig. 1 e, f). The overall frequency of plantlet regeneration through the steps of embryo germination, shoot formation and rooting on the same medium was 10.4 % (Fig. 1 g, h, i, j, k and l).

After 5 months following the transfer of the putatively transformed embryogenic clusters to the regeneration medium, the conversion rate (root and shoot growth) of plant development was also determined by differentiation into three morphological structures: total lack of any regeneration (structure I), abnormally developed germinating embryos (structure II), such as shoot and root deformations or the suppression of root induction and intact rooted plantlets (structure III) (Fig. 2 a, b and c). Of the germinating hygromycin resistant transgenic embryos, about 11 % showed normal shoot and root development compared to a conversion frequency of 76 % in the control plantlets. Seven regenerated lines (chi-4, 5, 9, 10, 14, 15 and 18) were used for further analyses. Each transgenic line was derived from a single somatic embryo and grew on hygromycin-containing media. The transgenic plants had no phenotypic abnormalities in comparison to the untransformed control plants.

Molecular and biochemical characterization of transgenic plants: Existence of the pGL2 (*CaMV-Ubi-Chi11*) gene in the regenerated grapevine plantlets was confirmed by PCR analysis using gene-specific primers which amplified a 350-bp fragment. The *hpt* (hygromycin phosphotransferase) gene was also detected by PCR with gene specific primers that amplified a 508-bp region. All seven transformants (chi-4, 5, 9, 10, 14, 15, and 18) were positive for the 508-bp *hpt* band (Fig. 3 a) but only four regenerants (chi-4, 9, 15 and 18) were confirmed to possess 350-bp region of the pGL2 (*CaMV-Ubi-Chi11*) gene (Fig. 3 b). There was no amplification observed in the untransformed plants. These four plants were studied subsequently.

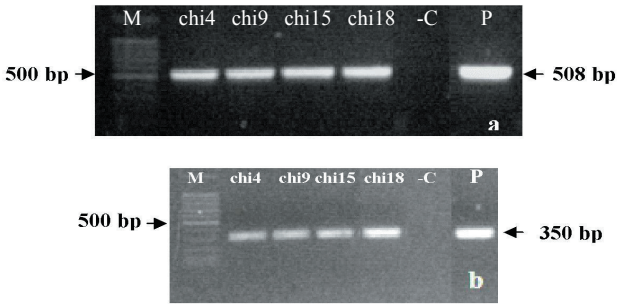


Fig. 3: PCR amplification of DNA isolated from transformed lines (**a**: hpt gene and **b**: pGL2 gene). Numbers indicate plant lines (4, 9, 15 and 18), *P*- Plasmid positive control; C: untransformed plant as negative control; *M*: 100 bp marker; arrow indicates amplicon size.

The integration of foreign genes and copy number pattern of integrated transgene in the nuclear genome of the PCR positive transgenic lines was confirmed by Southern hybridization. The genomic DNA was digested with *Pst*I restriction endonuclease enzyme in order to release the pGL2 (*CaMV-Ubi-Chi11*) cDNA-terminator cassette (~ 1.1 kb). The blot was probed with ^{32}P-dCTP labeled pGL2 (*CaMV-Ubi-Chi11*) cDNA. In all the four transgenic lines analyzed, the expected ~ 1.1-kb band was observed at the position corresponding to the chitinase gene within the genomes while it was absent in the untransformed control plant. DNA was also digested with *Sac*II, which has a single recognition site in the T-DNA region to determine the number of integration events and probed with ^{32}P-dCTP labelled *hpt* gene fragment. Single bands were seen in all the transgenic lines confirming the single copy integration in all four lines (Fig. 5 a and b).

We used RT-PCR analysis to check the expression of rice chitinase and actin genes. In order to study the expression of mRNA for pGL2 (*CaMV-Ubi-Chi11*) gene, total RNA was taken and RT-PCR analysis was performed from all five PCR positive lines using primers specific for the mRNA sequence of pGL2. A 290-bp amplified fragment of mRNA corresponding to the pGL2 (*CaMV-Ubi-Chi11*) transcript confirmed the expression of the rice chitinase. No amplification was observed in RNA samples isolated from untransformed plants but actin-specific transcript was present uniformly in all transformed and untransformed plants. RT-PCR results of all four independent transgenic lines (chi 4, 9, 15 and 18) are shown in Fig. 4.

Western blot analysis of representative lines employing polyclonal antibodies raised against bean chitinase showed the presence of a single prominent 35 kDa band corresponding to the expected size, indicating that the transgene is being expressed constitutively at a higher rate (Fig. 6). Higher chitinase activity was also detected in all transgenic plants expressing the pGL2 (*CaMV-Ubi-Chi11*)

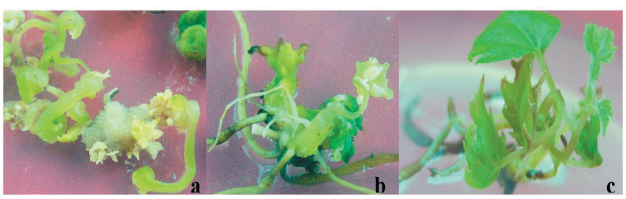


Fig. 2: Development of three morphological structures. **a**: structure I, no regeneration; **b**: Structure II, abnormal development with shoot or root deformations. **c**: Structure III, intact rooted plantlet.

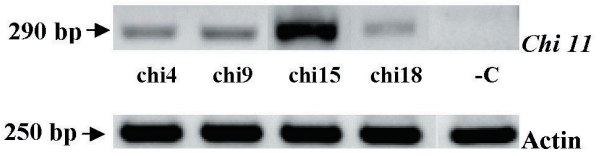


Fig. 4: Analysis of the plant lines for transgene by RT-PCR. The *Chi11* primers (290-bp amplicon) were used for amplification (Upper panel). RT-PCR with actin gene-specific primers (250-bp amplicon)was used as a control (Lower panel). Numbers indicate plant lines (4, 9, 15 and 18); C: untransformed.

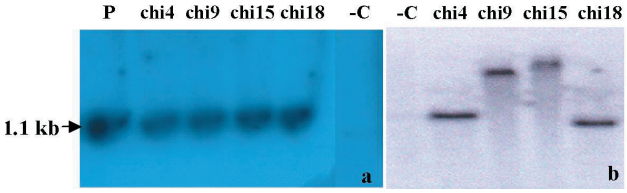


Fig. 5: Southern blot analyses of transgenic Pusa Seedless. Genomic and plasmid DNA digested with *Pst*I **a**: or *Sac*II **b**: were hybridized with PCR-amplified fragments of chitinase pGL2 (*CaMV-Ubi-Chi11*) and *hpt* genes derived probes. (Lanes: *C* - untransformed plant, *P* - plasmid, transgenic lines 4, 9, 15, and 18).

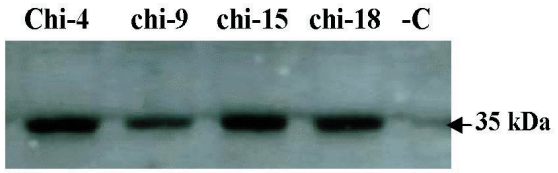


Fig. 6: Western-blot analyses. Equal amounts (25 µg) of soluble proteins were loaded in 12 % SDS-gels. Proteins isolated from leaf of transgenic lines were probed with antiserum to bean chitinase. Numbers indicate plant lines (4, 9, 15 and 18); C: untransformed plant used as a control.

gene investigated (chi4, chi9, chi15 and chi18) than in the non-transgenic plants. Lines 4 and 15 showed approximately two to three fold increase in the enzyme activity than non-transgenic plants, while lines 9 and 18 showed approximately one and half fold increase in the activity (Fig. 7).

Evaluation of powdery mildew resistance in transgenic plants: Detached leaves of transgenic plants were tested for resistance to the phytopathogenic fungus, *Erysiphe necator*. Both chi-4 and chi-15 lines showed disease-rating scores of 3.0 and 3.3 as an average score of three experiments, respectively, versus a score of 4.7 for the non-transformants (Tab. 1). These results indicate that the two transformants exhibited partial resistance to *E. necator*, which was sufficient to delay the spread of lesion areas of the disease. The degree of disease symptoms correlated well with the level of chitinase enzyme *i.e.* the transgenic plants with a higher level of chitinase activity tended to have a smaller total lesion area. Since the leaves used for the experiment were similar in the position and size, we also recorded the number of days required for the complete necrosis in each leaf. The results are shown in Tab. 2. Similar to the result of disease index, both chi-4 and chi-15 lines took longer period for the development of necrosis (Fig. 8).

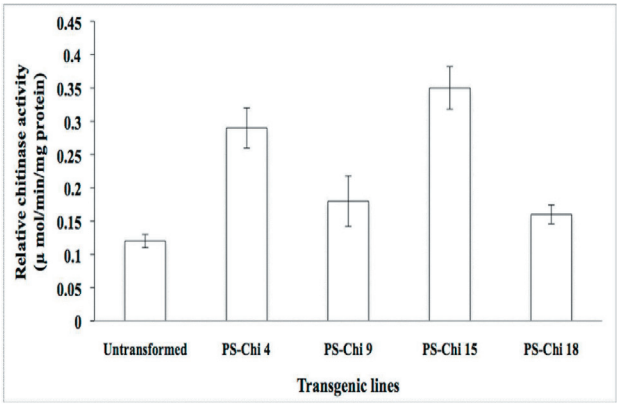


Fig. 7: A graph showing chitinase activity of different transgenic lines of 'Pusa Seedless'. Average values of three different experiments each in duplicates were plotted.

Table 1

Tolerance potential of transgenic grapevine plants to powdery mildew

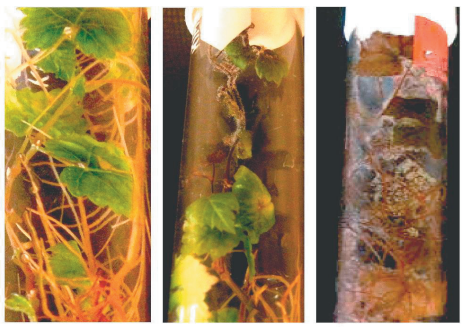
No. of lines	Disease rating scales for powdery mildew[a]
chi-4	3.0 ± 0.55[b]
chi-9	4.5 ± 0.46
chi-15	3.3 ± 0.58
chi-18	4.0 ± 0.49
untransformed	4.7 ± 0.51

[a] 0, symptoms; 5, very severe symptoms
[b] mean SD (±) of disease rating scale

Table 2

Number of days required for onset of disease and the complete leaf necrosis caused by powdery mildew on detached leaves from *chi11* transgenic lines

Plants	First symptoms (days)	Fully necrotic leaf (days)
Chi-4	9	24
Chi-9	6	18
Chi-15	8	25
Chi-18	7	20
Untransformed	3	14



a b c

Fig. 8: Infection on leaves of untransformed and chi-15 transgenic plants, 18 d after inoculation with powdery mildew spores. Necrotic lesions increased gradually on leaves of transgenic line chi-15 (**a** and **b**), whereas leaves of untransformed plants turned completely necrotic.

Discussion

It has previously been reported that several plant species expressing the rice chitinase gene conferred an enhanced resistance to fungal disease, *i.e.* resistance of rice against the Sheath Blight (Lin *et al*. 1995), transgenic indica rice variety Pusa Basmati 1 exhibits enhanced resistance to *Rhizoctonia solani* (Sridevi *et al.* 2003), genetically transformed pigeon pea for enhanced resistance to *Fusarium spp*. causing the wilt disease (Kumar *et al*. 2004) and transgenic taro resistance against *Sclerotium rolfsii* (He *et al*. 2008). Here, we present another illustration of enhanced resistance to powdery mildew in a transgenic grapevine. The present study shows that leaf-discs derived embryogenic calli could be transformed by *Agrobacterium*-mediated transformation harbouring the binary vector pGL2 (*CaMV-Ubi-Chi11*). These results demonstrate that embryogenic calli are well suited as target material for *Agrobacterium tumefaciens*-mediated transformation in grapes. The transformation process *via Agrobacterium* inoculation may cause necrosis of the tissue, which could be reduced by special treatments (Perl *et al.* 1996).

We have earlier observed that modification in medium along with manipulation in hormonal levels were required for proper germination and regeneration of transformants (Das *et al*. 2002). Using the same procedure the overall frequency of 10.4 % of hygromycin resistant plantlets regenerated through the steps of embryo germination, shoot formation and rooting could be achieved. It is known that the response of explants to regeneration is affected by genotype and developmental stage as well as endogenous growth regulators.

Molecular analyses involving PCR and Southern blotting indicate the integration of T-DNA into the grapevine genome. Four transformants (chi-4, 9, 15 and 18) were positive in Southern when probed with the pGL2 (*CaMV-Ubi-Chi11*) gene sequences. Presence of a single band in the Southern blotting experiments with DNA digested with *Sac*II, probed with the *hpt* gene sequences suggests single copy integration events. The difference detected using the two different restriction enzymes could be ascribed according to the restriction sites, where *Pst*I releases the 1.1-kb gene cassette while *Sac*II would cut once in the T-DNA region. Different sized bands among the transgenic lines indicate independent transformation events. The presence of rice chitinase gene transcripts in transgenic plants was demonstrated by RT-PCR analysis. Densitometry assessment gave the integrated density value (IDV) of each band. This shows that line chi-15 has a higher IDV than the rest. The higher level of transcript possibly results in more chitinase activity.

Expression of the pGL2 (*CaMV-Ubi-Chi11*) by Western blotting clearly detected a specific signal for the rice chitinase protein in the transgenic plants. Transgenic line chi-15 showed an approximately two fold increase in the chitinase enzyme activity while the transgenic lines chi-4, 9 and 18 showed approximately one and half fold increase in the enzyme activity and this could be correlated well with the degree of resistance to the pathogens. Previous reports have also shown that elevated chitinase activity in transgenic strawberry (Asao *et al*. 1997), rice (Nishizawa *et al*. 1999), tobacco (Emani *et al*. 2003).

Delays in the development of symptoms of fungal disease were observed in the transgenic plants expressing an exogenous chitinase gene (Broglie *et al*. 1991, Nishizawa *et al*. 1999, Yamamoto *et al*. 2000). We also observed that the symptoms of powdery mildew gradually progressed even on leaves of transgenic plants that exhibited high chitinase activity, so that the transgenic and non transgenic plants were indistinguishable when culture of the infected leaves was prolonged (> 18 d). The previous reports and the present study indicate that high expression of the chitinase gene confers improved tolerance to powdery mildew disease on grapevine plants, although the enhanced resistance is partial and quantitative.

In the present study, we used the *in vitro* inoculation method with detached leaves to evaluate the increased resistance against powdery mildew in transgenic plants. This method showed high accuracy for the detection of slight differences such as a delay in the development of disease symptoms, in fully controlled conditions, between the original nontransgenic plants and primary transformants having the same genetic background.

In conclusion, we demonstrate the successful transformation of leaf disc derived somatic embryos of *Vitis vinifera* 'Pusa seedless' and regenerated into plants. Following successful integration of the transferred rice chitinase gene pGL2 (*CaMV-Ubi-Chi11*) into the grapevine genome and expression of transferred gene, showed an increased tolerance to powdery mildew. Together, these findings suggest that the rice chitinase pGL2 (*CaMV-Ubi-Chi11*) gene could be utilized as a genetic source of disease resistance for breeding and improving crop species.

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