The Cloning of Potentially Useful Genes for Increased Resistance to Pest and Diseases in Crop Plants

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Introduction

Fungal pathogens cause heavy crops losses amounting to several billion dollars. Molecular biology of pathogenesis is an important field that has changed disease management strategy from chemical control to development of transgenic disease-resistant plants or "induced systemic resistant" plant. Several genes from either plants or microorganism (bacteria and fungi) encoding proteins with in vitro antifungal activity have been analysed. However only in a few cases it could be demonstrated that the observed *in vitro* antifungal activity correlated with in vivo protection in transgenic plants.

The defense responses of plant during infection with fungal pathogen include the inducible synthesis of a number of proteins, which, directly or indirectly, may participate in the active protection against the invading pathogen. Among these proteins are the hydrolytic enzymes, e.g. *chitinase* and β-1,3-*glucanase*. It has been well established that plant *chitinases* and β-1,3-*glucanase* have a potential to partially degrade fungal cell walls. The products formed are oligosaccharides, and it is possible that such oligosaccharides are perceived by the plant cell as signal, so-called elicitors to induce active defence responses.

The primary aim of this project is to isolate a chitinase gene from winged bean seeds. Chitinases hydrolyze the beta-1,4 linkages in chitin, a biopolymer of N-acetyl -D-glucosamine. Chitin is a cell wall component of many phytopathogenic fungal species. Because plants lack endogenous chitin, plant chitinases are thought to play an antifungal role. We are interested in identifying and characterising the natural defence system of local winged bean, and have decided to focus on the role of chitinases in the protection of local winged bean from pathogens. Winged bean was chosen in this study because it is unusually resistant to diseases and hence may be a potential source of isolating disease-resistant genes.

Materials and Methods

Plant Materials

The seeds of winged bean (which include seeds from various stages of bean development after fruiting) were obtained from Universiti Putra Malaysia. The other parts of winged bean plant were also collected for use in the expression study.

Isolation of RNA

Total RNA samples were isolated from plant tissue using a method described by Shultz *et al* (1994). Total RNA was isolated from 1 week to 6 weeks seed after fruiting and also from pod, stem, root and tuber.

RT-PCR Assay Procedure

For RT-PCR experiment, samples from 4 weeks-old developing seeds was used and the primer used were designed from the conserved region of *Nicotiana tabacum, Zea mays, Medicago sativa* and *Gossypium hirtusum.*

Construction of cDNA Library and Screening

A cDNA library was constructed using a Stratagene kit and the screening was carried out using a plaque lifts method. All procedures for construction and screening were carried out using a non-radioactive method (Boehringer Mannheim).

DNA Sequencing and Sequence Analysis

Sequencing was carried out using an automated sequencer and the result obtained was compared with genebank database. All sequences reported here were determined from both strands.

Northern and Southern Hybridization

Northern and Southern hybridization were according to Sambrook et al. (1990). For northern blotting, 25 μg of total RNA from a different tissue were separated electrophoretically and blotted onto Hybond-N+ membrane (Amersham) according to standard protocol. The probe was labelled using non-radioactive method. Stringent washing were carried out at room temperature using 2x SSC, 0.1%(w/v) SDS (5 min) and at 68°C using 0.2x SSC, 0.1% (w/v) SDS (twice for 15 min). For southern blotting 20 μg of total DNA was used and digested with *EcoR* I, *Hind* III, *EcoR* V, *pst I* and *Not* I. The same method was used for DNA transfer, hybridisation and washing step.

Results and Discussion

Reverse Transcriptase Polymerase Chain Reaction/RT-PCR

Two primers named CHRF and CHRR were synthesised from the conserved regions of chitinase class I and class IV. After PCR, 1.1 kb fragment was amplified and this fragment was then cloned into PCR blunt vector and transform to DH5α competent cells. This clone was named as CHRZ1 and then was sending for sequencing to identify the DNA sequences. The sequencing was carried out for both sides using M13 forward and reverse primers. The CHRZ1 sequence obtained was compared to other DNA sequences from EMBL database. The results indicated that 520 bp of CHRZ1 sequences showed more than 87% homology with chitinase class 1 from rice.

Assessment of Library Quality

The quality of the library was assessed and the results showed that the percentage of non-recombinant (blue

plaque) was low (1.92%) compared to the 10% of the upper limit and for PCR-based plaque screen, and the average size of inserts was more than 1 kb, indicating that good quality of cDNA library were obtained.

cDNA Library Screening and Isolation of Clone Containing Chitinase gene

The chitinase fragment, CHRZ1 from RT-PCR product was labelled as a probe and was prepared by digesting CHRZ1 DNA with *BamH* I and *Kpn* I purifying 490 bp fragment. Twelve strong positive signals were obtained after screening 250,000 plaques. After tertiary screening a few positive clone were excised from the Lambda ZAP vector and PCR using T3 and T7 primers to check the insert size. The largest insert (~1350 bp) was selected and sequenced. The sequencing was carried out for both ends and this clone was named as pCHRZP and it was found to encode for chitinase.

Nucleotide Sequence Analysis

The amino acid sequence of the putative chitinase gene was compared with chitinases from other plants, namely *P.vulgaris. Oryza sativa, Zea mays, Gossypium hirtusum* and *Hordeum vulgaree.* A search of the Gene Bank database revealed that the pCHRZP cDNA clone was more than 70% homologous to other chitinases from other plants. Recently, the cloning of a gene encoding chitinase from soy bean was reported, however, the winged bean chitinase exhibits a higher homology to *Oryza sativa* chitinase than to soy bean chitinase with 88% amino acid sequence identity. Homology to *P. vulgaris, Hordeum vulgare, Gossipium hirtusum* and *Zea mays* chitinases were 87%, 83%, 82% and 74% respectively. Based on the nucleotide and deduced amino acid sequences, pCHRZP clone apparently encoded a member of Class I Chitinase with the following features; a) a highly hydrophobic signal peptide with 48 amino acid; b) a cysteine-rich domain (also called the havein domain) in the N-terminal region.

Northern Hybridisation

Northern blot analysis was undertaken to characterise the stage at which CHRZP are expressed in winged bean tissue. In the case of seed samples, different stages of seed development were analysed, from 1 week to 6 weeks seed. The expression of CHRZP was undetectable in the earlier stage of seed (1-3 weeks) and is first noticed in 4 weeks of winged bean seed with the transcript size of approximately 1.2 kb. The expression level in 6-week-old seed was slightly lower than in 4 and 5 weeks old seeds. Expression was also detected in leave, tuber and pod with the same transcript size but not in root and stem.

Conclusions

A putative chitinase gene from developing winged bean seeds was successfully cloned and partially characterised. The gene appeared to encode a polypeptide belonging to a member of Class 1 Chitinases. Expression study revealed that the gene was expressed at least after the 4th weeks of seed development and continued until the 6th weeks. The gene was also expressed in tissues of leaf, pod and tuber but not in root and stem.

Benefits from the study

Chitinases, which catalyse the hydrolysis of the β-1,4-N-acetyl-D glucosamine linkages of the fungal cell wall polymer chitin, are involved in inducible defences of plants. The isolation and characterisation of potentially useful chitinase gene may pave the way for its incorporation into crop plants where the regeneration protocol have been established. It is therefore necessary that transformation system for local crop plants is established so that the isolated gene can be introduced in the hope of conferring increased resistance to diseases caused by pathogens.

Literature cited in the text

None.

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