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Cloning and Sequencing of Strawberry Chitinase Gene CHI2

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Abstract: Two genes fragments from cultivated strawberry (Fragaria x ananassa cv. Toyonaka and cv. Akihime) genomic DNA were isolated by PCR amplification. The study showed that these genes were 1575 bp in length and contained a 988 bp Open Reading Frome (ORF) and 2 extron and 1 intron. The homology of nucleotide and deduced amino acid were 99.05 and 98.56%, respectively. Sequecing revealed that the 2 genes encoded a class chitinase. BLAST analysis showed that the homology of deduced amino acid between strawberry and other species's CHI2 gene were about 50%. The homology tree showed that it obviously distinguished from the others.

Key words: Strawberry, chitinase gene, cloning, sequencing, amino acid, CHI2

INTRODUCTION

Chitin, a β-(1, 4) linked homopolymer of N-acetylglucosamine (GlcNAc), is the 2nd most abundant polysaccharide in nature and a principal structure component in the cell walls of fungi and exoskeleton of invertebrates (Warren, 1996). Chitinases (EC 3.2.1.14) in higher plants catalyze the hydrolysis of chitin into oligomer or monomer (Raikel *et al*., 1993). In plant cells, chitinase is induced by pathogenic attack (Rasmussen *et al*., 1992), wounding (Ming-Mei *et al*., 1995) and plant hormones (Mauch and Staehelin, 1989), as well as by a number of other environmental stresses (Bowles, 1990). Recent studies also indicated that chitinases are one of the major classes of Pathogenesis Related (PR) proteins in plants, which are believed to play important roles in plant defense against infection by pathogens (Melchers *et al*., 1994; Neuhaus, 1999). Transgenic tobacco and potato plants developed by insertion of a chitinase gene from Trichoderma harzianum have been shown to exhibit either high tolerance or complete resistance to the foliar pathogens Alternaria alternate, Alternaria solani, *Botrytis cinerea* and the soil borne pathogen Rhizoctonia solani (Lorito *et al*., 1998).

Strawberry (Fragaria x ananassa) is one of the most economically and biologically important fruit trees in the world. In strawberry production, the polyphagous grey mould fungus (*Botrytis cinerea*) is undoubtedly the most important pathogen causing fruit rot. In organic or unsprayed fields, the losses due to fruit rot can be up to 55% (Daugaard, 1999). To increase strawberry cultivar resistant to B. cinerea is one of the main targets in strawberry breeding. In this study, the isolation and characterization of a chitinases gene from strawberry 'Toyonaka' were reported as a part of a wider study to enhance the resistance of strawberry to fungal diseases.

MATERIALS AND METHODS

DNA preparation and primer design: Genomic DNA was obtained from young leaves by the method of Pich and Schubert (1993). The primer used for isolating strawberry chitinases gene was designed based on the sequence from Khan and Shih (2004) and the conserved regions

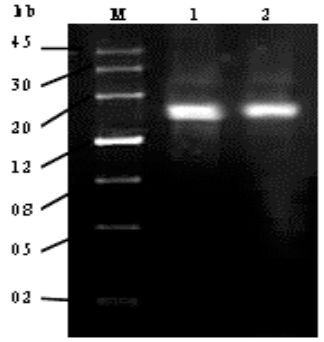

Fig. 1: Agarose gel electrophoresis of PCR products. M indicates the molecular weight standard; lane 1 indicates the PCR products correspond to Toyonaka; lane 2 indicates the PCR products correspond to Akihime

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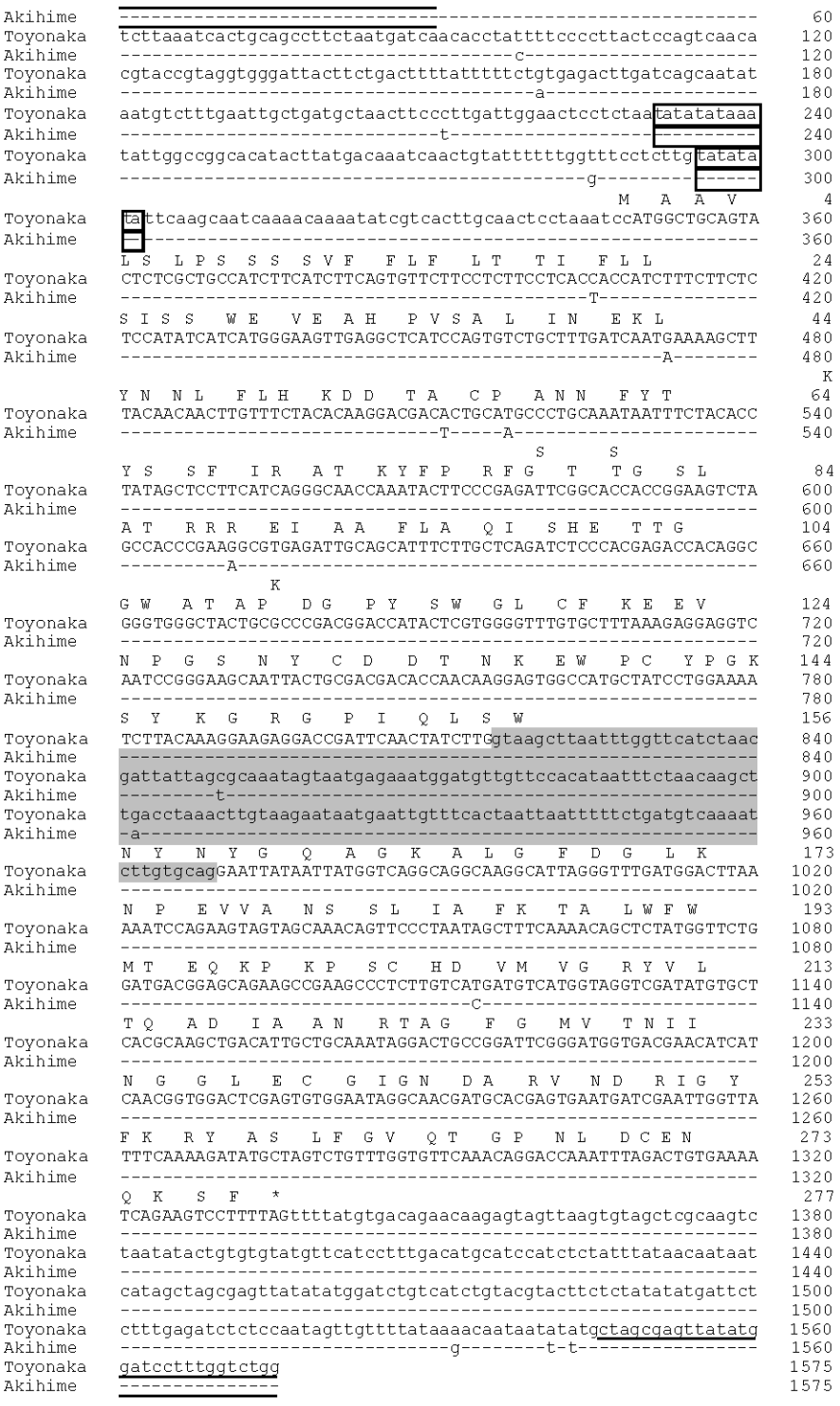

Fig. 2: Nucleic acid sequence of chitinases gene (upper) and its deduced amino acid sequence of Toyonaka and Akihime. The underline indicate primer locations; the lowercase characters indicate noncoding region; dashed areas indicate the intron; the uppercase characters indicate coding region; indicate the stop codon; rectangle indicate the TATA box

Table 1: Comparison of the derived amino acid sequences of the CHI2 genes (%)

Amino acids	Toyonaka	Akihime	AF147091	AF420225	AF000966	AF141372	AF241538	AY544781	AY775335	L37876
Toyonaka	100.0									
Akihime	98.6	100.0								
AF147091	98.9	98.2	100.0							
AF420225	51.9	51.9	52.3	100.0						
AF000966	51.8	51.8	52.2	67.3	100.0					
AF141372	55.3	55.6	55.6	66.9	68.5	100.0				
AF241538	49.4	49.4	49.8	59.5	52.8	56.2	100.0			
AY544781	50.0	49.6	50.0	59.6	56.7	59.4	50.4	100.0		
AY775335	44.4	44.4	44.8	60.7	56.4	58.5	48.8	53.4	100.0	
L37876	52.7	52.7	53.1	67.7	59.2	64.7	53.3	57.0	51.4	100

of chitinases genes in GenBank from NCBI web site (www.ncbi.nlm.nih.gov). The designed primer sequences were shown as follows:

Fa-1F: 5'-ACGGAGTCAACAATCAAGGTACCGACAC GA -3'
Fa-1R: 5'-CCAGACCAAAGGAT CCATATAACTCGCTAG-3'

PCR amplification and cloning of PCR products: PCR was performed with a 25 μL reaction mixture containing 20 ng of strawberry genomic DNA, 800 μM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer without Mg^{2+}, 0.4 μM of each primers and 4 unit of Taq DNA Polymerase using the following profile: 94°C for 3 min, then 30 cycles of 94°C for 1 min, 65°C for 1 min, 70°C for 2 min and finally 72°C for 10 min. The PCR products were analyzed on a 1.2% agarose/EtBr gel and the corresponding DNA bands were recovered and then cloned into the pMD18-T vector (Takara) for sequencing. Sequence analysis was performed using the software DNAMAN (Version 3.0, Lynnon BioSoft).

Isolation of strawberry chitinases gene: In this study, a pair of specific primers was used to amplify DNA fragment from strawberry genomic DNA. PCR amplification generated DNA products with the expected size of about 1.5 kb (Fig. 1). Two putative chitinase DNA fragments were identified and cloned into pMD18-T vector (TaKaRa) and then transformed into *E. coli* JM109 competent cells. Both fragments were sequenced with Big Dye Terminators using a PRISM 377 Sequencer (PE Applied Biosystems, Foster City, California, USA) and were designated as CHI1 and CHI2, respectively.

Structural analysis of strawberry chitinases gene: Analysis of the both sequences revealed that the DNA of strawberry chitinases gene are 1575 bp in length and contain a 988 bp Open Reading Frame (ORF). The coding region of CHI1 and CHI2 are divided into 2 exons interrupted by a 154 bp intron. The splice junctions were determined by comparing the sequence of FaCHI 2-1 with the sequence of a corresponding cDNA (GenBank accession number AF 420226). The intron showed a high AT content (67.9%) characteristic of dicotyledonous plant introns. The intron has good 5' and 3' splice site consensus CAG-GT and ACAG-GT, respectively. Two

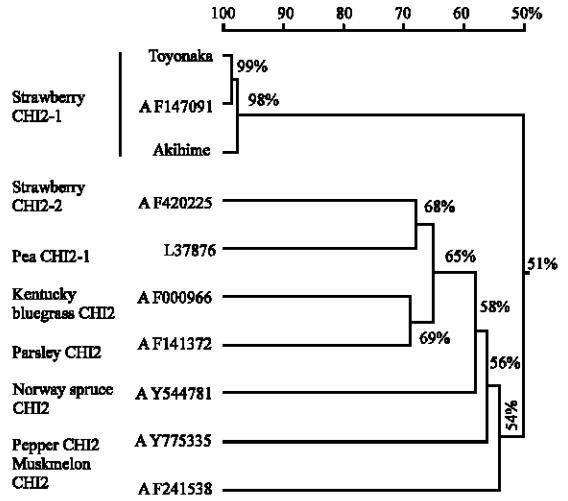

Fig. 3: Homology tree of the derived amino acid sequences of the CHI2 genes

overlapping TATA-like sequences are present in the 348 bp 5'-flanking region. These sequence elements are located at -230 (TATATATAA) and -294 (TATATATA) positions upstream of the predicted translation start site. Three weak polyadenylation signals are present in the 239 bp 3'-flanking region downstream of the stop codon (Fig. 2).

Comparison results between the sequence of the CHI1 and CHI2 indicated only 15 different bases were present in both genes (Fig. 1). CHI1 shows the highest nucleic acid sequence homology to CHI2 with 99.1% identity and 98.6% similarity its deduced amino acid sequence. The translation product of CHI1 and CHI2 are predicted to have a signal peptide of 33 amino acids and a mature protein of 277 amino acids with a molecular mass of 30.6 kDa and an isoelectric point of 8.53. CHI1 and CHI2 shows the highest sequence homology to a strawberry class II chitinase gene (AF147091) with 98% identity, but shows the lower sequence homology to other plants class II chitinase gene with 50% similarity, such as pea (L37876), kentucky bluegrass (AF000966), pepper (AY775335), with homologies of 52.7, 51.8 and 44.4%, respectively (Table 1). The homology tree showed that strawberry class II chitinase gene obviously distinguished from the others (Fig. 3).

RESULTS AND DISCUSSION

Plant chitinases are pathogenesis-related proteins, which are believed to play important roles in plant defense against infection by pathogens. Molecular cloning of chitinases from various plants has facilitated the classification of these proteins. All plants examined to date contain multiple chitinase isozymes, which have been divided into 7 classes (classes IBVII) on the basis of their structural properties (Neuhaus, 1999).

Several class I chitinases have been shown to inhibit fungal growth *in vitro* (Sela-Buurlage *et al*., 1993). On the other hand, a class II chitinase from tobacco (Sela-Buurlage *et al*., 1993) showed no detectable *in vitro* inhibitory activity.

However, transgenic tobacco (Jach *et al*., 1995) and wheat plants (Oldach *et al*., 2001) expressing a barley class II chitinase also showed enhanced resistance to fungal infection.

Furthermore, incorporation of a class II chitinase for producing disease-resistant transgenic plants may be a better strategy since the Chitin-Binding Domain (CBD) of class enzymes has been implicated in the allergenic reactions. Class II chitinases, which lack the CBD, were shown not to elicit allergenic reactions (Poash *et al*., 1999; Diaz Perales *et al*., 1998).

In this study, 2 DNA fragment were obtained from strawberry genomic DNA. The genes were classified as class II chitinase genes based on their structures. Both genes are similar to other intron-containing plant chitinase genes. The locations of introns are conserved in these genes. However, the lengths of introns vary considerably, introns in strawberry chitinase genes are 125-169 bp in length. The deduced amino acid sequence of class II chitinase genes contained 2 highly conserved region, AFLAQI (/T)SHETTGG and FKTAL (/I)WFWWT and the conservation of 3'-flanking region is >5'-flanking region.

The homology tree showed that strawberry class II chitinase gene obviously distinguished from the others and may have different disease activity, but whether it means that it has a unique feature on the antimicrobial activity remains to be further study.

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