

**CHARACTERIZATION OF CHITINASE ACTIVITY AND GENE  
EXPRESSION IN MUSKMELON SEEDS**

by

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## **ABSTRACT**

Chitinase has been suggested to play a role in defense mechanisms. In this study, the activity and expression of chitinase in muskmelon seeds were investigated. Multiple chitinase isoforms were detected in muskmelon seeds from early development through radicle emergence. One acidic and three basic chitinase isoforms were detected in developing seeds at 40 days after anthesis (DAA). Both acidic and basic chitinase isoforms were detected in endosperm tissue during seed imbibition and after radicle emergence. Basic chitinase isoforms, but not acidic isoforms, were detected in embryo tissue. Basic chitinase isoforms were also detected in the embryonic axis or radicle tissue. Taken together, these observations indicate that chitinases are regulated developmentally and in a tissue-specific manner in muskmelon seeds. Therefore the potential function of chitinases in muskmelon seeds is discussed.

Two complete cDNAs, *Cmchi1* and *Cmchi2*, and a partial genomic clone of *Cmchi2* have been isolated from muskmelon seeds. *Cmchi2* gene has two introns in the

coding region while *Cmchi1* is intronless. *Cmchi1* cDNA encodes a class III chitinase while *Cmchi2* cDNA encodes a class II chitinase. *Cmchi1* and *Cmchi2* proteins might be targeted to secretory pathways because they possess signal peptides.

Southern blotting suggested that there is at least one additional gene similar to *Cmchi1* in the muskmelon seed genome, while there is only one copy of *Cmchi2*. Northern blotting analysis showed that both *Cmchi1* and *Cmchi2* are expressed in the radicle tissue at the time of radicle emergence. This indicates that the expression is regulated developmentally and in a tissue-specific manner. Salicylic acid (SA) and benzothiadiazole (BTH) stimulated the expression of *Cmchi1* but not *Cmchi2* in seeds after radicle emergence, indicating that SA might be involved in inducing the expression of *Cmchi1*, while a different signal might be involved in triggering the expression of *Cmchi2*.

The protein encoded by *Cmchi1* cDNA was expressed in *E.coli*. It did not show any enzymatic activity. Western blotting using an antibody raised against the class III chitinase protein in cucumber was inconclusive, as this antibody recognized the purified *Cmchi1* fusion protein and other unknown proteins isolated from the embryonic axis or the radicle tissue.

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## TABLE OF CONTENTS

TITLE AND SIGNATURE .....	I
ABSTRACT.....	II
ACKNOWLEDGEMENTS.....	IV
TABLE OF CONTENTS.....	V
LIST OF TABLES.....	VII
LIST OF FIGURES .....	VIII
CHAPTER ONE.....	1
LITERATURE REVIEW .....	1
INTRODUCTION .....	21
CHAPTER TWO .....	25
CHARACTERIZATION OF CHITINASE ACTIVITY IN MUSKMELON SEEDS.....	25
MATERIALS AND METHODS .....	25
RESULTS .....	28
FIGURES.....	32

CHAPTER THREE .....	42
CHAPTER THREE .....	43
CLONING OF CLASS II AND CLASS III CHITINASE GENES EXPRESSED IN GERMINATED MUSKMELON SEEDS .....	43
MATERIALS AND METHODS .....	43
RESULTS .....	56
FIGURES.....	64
CHAPTER FOUR.....	82
CHAPTER FOUR.....	83
CHARACTERIZATION OF <i>CMCH11</i> AND <i>CMCH12</i> EXPRESSION IN GERMINATED MUSKMELON SEEDS AND EXPRESSION OF <i>CMCH11</i> IN <i>E. COLI</i> .....	83
MATERIALS AND METHODS .....	83
RESULTS .....	92
FIGURES.....	96
CONCLUSIONS.....	126
REFERENCES .....	133
VITA.....	159

## LIST OF TABLES

Table 3-1. Primers and templates for amplifying 3' and 5' end of <i>Cmchi1</i> cDNA.....	51
Table 3-2. Primers and templates for amplifying 3' and 5' end of <i>Cmchi2</i> cDNA.....	52
Table 3-3. Proteins with sequence identity to <i>Cmchi1</i> protein. ....	75
Table 3-4. Proteins with sequence similarity to <i>Cmchi2</i> protein.....	78
Table 5. Summary of chitinase activity and different isoforms present in developing seeds, mature seeds, seeds during imbibition and after radicle emergence.....	131
Table 6. Summary of gene expression in developing muskmelon seeds, seeds during incubation with water, SA, or BTH at different times and in different tissues of mature plants.....	132

## LIST OF FIGURES

Figure 2-1. Gel diffusion assay of chitinase activity from muskmelon seeds. ....	33
Figure 2-2. Gel diffusion assay of chitinase activity in developing and mature muskmelon seeds. ....	34
Figure 2-3. Native-PAGE and activity staining of acidic chitinase isoform from muskmelon seeds at different developmental stages .....	35
Figure 2-4. Native-PAGE and activity staining for basic chitinase isoforms from muskmelon seeds at different developmental stages .....	36
Figure 2-5. Native-PAGE and activity staining for acidic chitinase from endosperm tissue .....	37
Figure 2-6. Native-PAGE and activity staining for basic chitinase from endosperm tissue .....	38
Figure 2-7. Native-PAGE and activity staining for acidic chitinase from embryo tissue	39
Figure 2-8. Native-PAGE and activity staining for acidic chitinase isoform from different sections of endosperm tissue .....	40
Figure 2-9. Native-PAGE and activity staining for basic chitinase isoform from muskmelon embryo tissue.....	41
Figure 2-10. Native-PAGE and activity staining for basic chitinase isoforms from embryonic axis (4, 12 h) or radicle tissue (24, 32 and 48h).....	42
Figure 3-1. RT-PCR products amplified from RNA isolated from germinated muskmelon	

seeds.....	65
Figure 3-2. PCR products for both putative <i>Cmchi1</i> and <i>Cmchi2</i> cDNA generated by the 3'RACE procedure.....	66
Figure 3-3. PCR products for <i>Cmchi1</i> generated by the 5' RACE procedure.....	67
Figure 3-4. PCR products for <i>Cmchi2</i> generated by the 5' RACE procedure .....	68
Figure 3-5. The full length of <i>Cmchi1</i> cDNA.....	69
Figure 3-6. The full length of <i>Cmchi1</i> amplified from cDNA or genomic DNA template .....	70
Figure 3-7. The full length of <i>Cmchi2</i> cDNA amplified from the cDNA template and the partial genomic <i>Cmchi2</i> PCR product .....	71
Figure 3-8. Complete cDNA sequence of <i>Cmchi1</i> and deduced amino acid sequence ..	72
Figure 3-9. Complete cDNA sequence of <i>Cmchi2</i> and deduced amino acid sequence ....	73
Figure 3-10. Partial genomic sequence of <i>Cmchi2</i> .....	74
Figure 3-11. Phylogenetic tree of <i>Cmchi1</i> protein with other related chitinases from databases .....	76
Figure 3-12. Amino acid sequence alignment of <i>Cmchi1</i> against other known class III chitinases.....	77
Figure 3-13. Phylogenetic tree of <i>Cmchi2</i> protein and other related chitinases from databases .....	79
Figure 3-14. Amino acid sequence alignment of <i>Cmchi2</i> against other known class II chitinases.....	80

Figure 3-15. Southern blot of <i>Cmchi1</i> encoding class III chitinase in germinated muskmelon seeds .....	81
Figure 3-16. Southern blot of <i>Cmchi2</i> encoding class II chitinase in germinated muskmelon seeds .....	82
Figure 4-1. Northern blot of analysis of <i>Cmchi1</i> (A) and <i>Cmchi2</i> (B) gene expression in muskmelon seeds during or after germination.....	97
Figure 4-2. Northern blot analysis of both <i>Cmchi1</i> and <i>Cmchi2</i> gene expression in radicle tissue and the cotyledon and endosperm.....	98
Figure 4-3. Northern blot analysis of <i>Cmchi1</i> or <i>Cmchi2</i> gene expression in different tissues and germinated muskmelon seeds.....	99
Figure 4-4. Northern blot analysis of <i>Cmchi1</i> gene expression in muskmelon seeds imbibed in water (control) and BTH or SA (treatment) .....	100
Figure 4-5. Northern blot analysis of <i>Cmchi2</i> gene expression in muskmelon seeds imbibed in water (control) and BTH or SA (treatment) .....	101
Figure 4-6. SDS-PAGE of <i>Cmchi1</i> fusion protein samples prepared in the pilot experiment and proteins resulting from renaturing and purification steps .....	102
Figure 4-7. SDS-PAGE of the purified fusion protein and renatured fusion protein prior to purification.....	103
Figure 4-8. SDS-PAGE of the fusion protein samples cleaved by Factor Xa .....	104
Figure 4-9. SDS-PAGE of protein samples from different sources.....	105

# CHAPTER ONE

## LITERATURE REVIEW

### Definition and biochemical properties

Chitin is a soluble, linear,  $\beta$ -1, 4-linked polymer of N-acetylglucosamine (NAG). Chitin is a common constituent of many organisms and is found in fungal cell walls, insect exoskeletons, and shells of crustaceans (Cabib, 1987). All of these chitin-containing organisms produce chitinase. Some other organisms, such as bacteria and higher plants, do not contain chitin but do produce chitinases. Chitinases (EC 3.2. 1. 14) are enzymes catalyzing the cleavage of a bond between C1 and C4 of two consecutive NAG monomers of chitin. The presence of chitinase was first described in 1911 by Bernard who found a thermosensitive and diffusible antifungal factor in orchid bulbs and in 1929 by Karrer and Hofmann who discovered chitinase in a snail (reviewed by Flach et al., 1992).

There are two kinds of chitinases: endochitinases and exochitinases.

Endochitinase cleaves chitin randomly at internal points within the polymer, releasing soluble, low-molecular weight multimers of NAG such as chitotetraose and chitotriose and the dimer, di-acetylchitobiose, which predominates. Exochitinase hydrolyzes chitin

by releasing di-acetylchitobiose, with no mono or oligo-saccharides formed. Other enzymes related to chitinase, such as  $\beta$ -N-acetylglucosaminidases and chitobiases, have also been characterized. Chitobiase hydrolyzes chitobiose.  $\beta$ -N-acetylglucosaminidase is defined as an enzyme releasing NAG monomers from oligomers (Flach et al., 1992).

Most plant chitinases isolated to date are endochitinases. However, several studies have shown that some plants have exochitinases. For example, one chitinase purified from sugar beet (Nielsen et al., 1993), two chitinases from carrot (Kurosaki et al., 1989), and three chitinases from *Hevea brasiliensis* (Martin, 1991) showed exochitinase activity. Some plant endochitinases also show some degree of lysozyme activity (EC 3. 2. 1. 17) corresponding to the cleavage of a glycosidic bond between the C1 of N-acetylmuramic acid and the C4 of N-acetylglucosamine in the bacterial peptidoglycan (Jolles et al., 1984; Majeau et al., 1990; Mauch et al., 1988a; Minic et al., 1998; Subroto et al., 1999). According to Beintema et al. (1996), all lysozymes have chitinase activity, but not all plant chitinases are lysozymes. All plant chitinases are relatively small proteins with molecular weights ranging from 25,000 to 40,000 kD. Chitinases typically exist as monomers; however, one study showed that at least one endochitinase from seeds of Job's tears (*Croix lachrymosa-jobi*) dimerizes (Ary et al., 1989).

Chitinases are classified as acidic or basic proteins based on isoelectric points. They may contain one putative chitin-binding domain in addition to the catalytic domain.

One study suggested that a chitinase from *Brassica* contains two chitin-binding domains in addition to its catalytic domain (Zhao et al., 1999).

While most chitinases are not known to be glycoproteins, chitinases from yam (Tsukamoto et al., 1984), bean (Margis-Pinhiero et al., 1991), and carrot (De Jong et al., 1992) are glycoproteins. A class IV chitinase from sugar beet is apparently glycosylated with xylose (Nielsen et al., 1994).

The catalytic function of chitinase appears to require no cofactors. This is supported by the evidence from Jeuniaux's study (1966) in which chitinase activity was not abolished by dialysis. The optimal pH for chitinase activity varies depending on the species but is usually acidic. For example, using chitin as the substrate, the in vitro pH optimum for a yam chitinase is 3.8 (Tsukamoto et al., 1984) and for a bean leaf chitinase is 6.5 (Boller et al., 1983). However, there are two chitinases from yam and each has two pH optima, one acidic and one basic (Tsukamoto et al., 1984).

### **Occurrence and classification of plant chitinases**

Plant chitinase proteins have been purified and studied in many dicot and monocot plant species including *Arabidopsis thaliana* (Samac et al., 1990; Verburg and Huynh, 1991), banana (Clendenen et al., 1998), bean (Awade et al., 1989; Boller, 1988), winged bean (Esaka and Teramoto, 1998), cucumber (Metraux et al., 1989), garlic (Van Damme et al., 1993), Job's tears (Ary et al., 1989), maize (Huynk et al., 1992; Wu et al., 1994), orange (Osswald et al., 1994), pokeweed (Yamagami et al., 1998), pea (Chang et

al, 1995), potato (Gaynor, 1988; 1989; Laflamme and Roxby, 1989), rice (Huang et al., 1991; Zhu and Lamb, 1991; Nishizawa and Hibi, 1991; Nishizawa et al., 1993; Nagasaki et al., 1997), soybean (Yeboah et al., 1998), sugar beet (Fleming et al., 1991; Nielsen et al., 1993), tobacco (Lawton et al., 1992; 1994), tomato (Danhash et al., 1993), yam (Araki et al., 1992a; 1992b), and wheat (Ride and Barber, 1990). Chitinases have been found in a wide variety of plant organs or tissues, including leaves, stems, roots, flowers, fruits, tubers, seeds, embryos, cotyledons, mesophyll protoplasts, and epidermal cells.

Based on amino acid sequence similarity of chitinases from various organisms, five classes of chitinases have been proposed. These classes can be grouped into two families of glycosyl hydrolases, family 18 and 19 (Henrissat et al., 1993; Hamel et al., 1997). Three distinct structural classes were first proposed by Shinshi et al. (1990). Class I chitinases are basic and have a N-terminal, cysteine-rich domain that has been implicated in chitin binding due to its homology with a polypeptide from *Hevea latex* that has chitin-binding properties. Most class I chitinases have a C-terminal extension that presumably targets them to the vacuole (Neuhaus et al., 1991b). Class I chitinases are found in both monocots and dicots. Class II chitinases are generally acidic and homologous to the class I chitinases within the catalytic domain but lack the N-terminal, cysteine-rich domain and the C-terminal extension. Not all class II chitinases are acidic. A chitinase from tobacco is basic and shows greater sequence identity to tobacco class I chitinase, but it lacks the N-terminal cysteine-rich domain and the C-terminal extension (Ohme-Takage et al., 1998). This class II chitinase is considered to be a putative ancestor

of basic class I chitinase. Class II chitinases are mainly found in dicots and are secreted into the extracellular spaces. Class III chitinases have no sequence similarity with class I and II chitinases but share high amino acid sequence similarities with a bifunctional chitinase-lysozyme from *Hevea brasiliensis* (Meins et al., 1992).

Collinge et al. (1993) proposed a class IV chitinase that contains the cysteine-rich domain and a conserved catalytic domain. However, it differs from class I chitinases because of its smaller size due to four deletions in the catalytic domain (Collinge et al., 1993). Melchers et al. (1994) found some endochitinase activity in tobacco, and these endochitinases could not be placed in class I-IV chitinases. They share some similarities with bacterial exochitinases and are designated as class V chitinases.

It has been suggested that chitinases from class I, II, and IV are of plant origin and make up the family 19 glycosyl hydrolases (Hamel et al., 1997). Class III chitinases are mainly plant and fungal in origin (Hamel et al., 1997). Class III and V make up the family 18 glycosyl hydrolases (Henrissat et al., 1991; 1993), which are structurally unrelated to family 19 (Meins et al., 1992). Phylogenetic analysis of chitinases from class I, II, and IV suggested a larger evolutionary distance between chitinases of class IV and those of classes I and II (Hamel et al., 1997).

Not all chitinases fall into the classification scheme mentioned above. A gene encoding a chitin-binding protein from elderberry consists of a N-terminal domain that has high sequence similarity to other class I chitinases, while the C-terminus is most

closely related to other class V chitinases (Van et al., 1999). The gene product is considered to be a hybrid between class I and class V chitinases.

Beintema (1994) pointed out that the above structural classification of chitinases is unsatisfactory and needs to be replaced by one that is more evolutionarily correct. He indicated that features like the presence of a N-terminal chitin-binding domain and vacuolar targeting signals, which are important in the structural classification, are not very useful for classification purposes. Many chitinases sequenced and characterized later did not fit into this classification. Beintema suggested that an evolutionary classification based on sequence similarity with a high weight on shared deletions in the sequences would be better than the structural classification of chitinases. He proposed the adoption of the designation b-type (class I, II and IV) and h-type (class III and V) chitinases, respectively.

### **Subcellular localization**

The localization of some chitinases has been determined by immunocytochemistry as well as cell fractionation methods (Fink et al., 1988; Flemming et al., 1991; Mauch and Staehelin, 1989; Wubbon et al., 1992). These studies indicated that chitinases could be deposited either in vacuoles or in the apoplast. Studies on tobacco class I chitinases have shown that a short C-terminal extension of about six amino acids is both necessary and sufficient for vacuolar targeting (Neuhaus et al., 1991b). This C-terminal extension

sequence is also found in other basic vacuolar chitinases from bean, potato, poplar, and *Arabidopsis* (Broglie et al., 1986; Gaynor, 1988; Parsons et al., 1989; Samac et al., 1990).

Some chitinases are also synthesized without the C-terminal sequences. This is the case for class IV chitinases from sugar beet and rape and some class I chitinases from pea, rice, and barley (Huang et al., 1991; Mikkelsen et al., 1992; Rasmussen et al., 1992a; Swegel et al., 1989; Vad, 1991). This indicates that these chitinases may be synthesized for secretion to the apoplast. A class IV chitinase from sugar beet was found to be deposited in the apoplast, which supports this hypothesis (Mikkelsen et al., 1992).

Chitinases designated for vacuoles can be secreted into the medium of cultured tobacco cells (Kunze et al., 1998). Vacuolar class I chitinase in tobacco suspension cultures accumulated in the medium within one week after subculturing. The secretion can be enhanced in the presence of 2, 4-dichlorophenoxyacetic acid (2, 4-D).

### **Regulation of chitinase activity and gene expression in plants**

Chitinases are found to be constitutively expressed in *Hevea* (Martin et al., 1991). The latex of *Hevea* contains high levels of chitinase. Papaya latex is a rich source of a class II chitinase (Azarkan et al., 1997). Potato also constitutively expresses chitinases (Beerhues et al., 1990).

Chitinase activity varies throughout a healthy plant in a developmental and tissue-specific manner. Some organs, such as roots and flowers, consistently express higher

chitinase activity than other organs. Chitinase found in tobacco roots can account for as much as 4% of the total soluble proteins (Shinshi et al., 1987). Chitinase expression in leaves is regulated mainly by leaf age. For example, acidic chitinase activity was detected in apical leaves but not in senescent tobacco leaves (Trudel et al., 1989). Expression of chitinase in seeds may be regulated by developmental stages. Chitinase could be detected in young seeds but not in mature plants. For example, one of the basic chitinases found in cucumber seeds almost completely disappeared 3 days after germination (Majeau et al., 1990). An acidic chitinase is specifically expressed in developing soybean seeds but not in leaves or stems (Yeboah et al., 1998).

Abiotic agents such as chitosan, ethylene, ozone, wounding, polysaccharides, salicylic acid, salt solution, and UV light can induce higher expression of chitinases in plants (Zhang and Punja, 1994; Ishige et al., 1993; Ernst et al., 1992; Esaka et al., 1993; Staehelin et al., 1994b; Siefert et al., 1994; Cohen et al., 1994; Margis-Pinheiro et al., 1993). Biotic agents such as bacteria, insects, fungi, viruses, and fungal cell wall fragments can also induce the expression of chitinases in plants (Meins and Ahl, 1989; Roberts et al., 1992; Wubben et al., 1994; Lawton et al., 1992; Kendra et al., 1989). More detailed information about induction of chitinase expression by abiotic agents and some biotic agents can be found in reviews by Graham (1994) and Flach et al. (1992). The following discussion will focus on the induction of chitinase expression by pathogenic infections

A number of studies have shown that the infection of a host plant by a pathogen initiates a complex series of biochemical events that includes the eliciting of defense responses. Chitinase is one of the biochemical compounds that are frequently found to be up-regulated. Studies of chitinases from bean, cucumber, pea, potato, sugar beet, tomato, and tobacco have shown that the expression of chitinases is induced dramatically after infection (Daugrois et al., 1990; Zhang and Punja, 1994; Dumas-Gaudot, 1994; Schroder et al., 1992; Rousseau-Limouzin and Fritig, 1991; Wubben et al., 1994; Meins and Ahl, 1989). Induction usually occurs strongly at the point of infection and drops rapidly as the distance from the infection site increases (Dore et al., 1991). The strength of the induction of chitinase activity also depends on the pathogenicity and compatibility between the host and pathogens (to be reviewed later). When the first leaves of cucumber plants were infected with *Colletotrichum lagenarium*, there was a 600-fold increase in chitinase activity in the infected areas (Mettraux and Boller, 1986), while the induction of chitinase in the uninfected area of the same leaf was only increased about 60-fold. Second leaves from the same plant showed an even smaller increase in chitinase activity but still exhibited higher level than that of the control plants. This also indicates that the induction not only occurs in the infected area but also can spread to adjacent tissues, resulting in a systemic acquired resistance that may enable the plant to protect itself from secondary infection. For example, cucumber plants became more resistant to *Colletotrichum lagenarium* infection after leaves were infected with tobacco necrosis virus (TNV), and the resistance was also correlated with an increase in chitinase activity

(Metraux and Boller, 1986). In tobacco, the accumulation of chitinase correlates with the acquisition of systemically induced resistance (Ye et al., 1990). However, chitinase is not always a part of systemic resistance response. Metraux et al. (1991) showed that *Arabidopsis* acquired systemic resistance after treated with salicylic acid by increasing the level of PR-1, PR-2 and PR-5 proteins.

The induction of chitinase expression can last several days. Chitinase activity from cucumber cotyledons infected with *Colletotrichum lagenarium* increased steadily to a maximum after 4 days and then declined to about 80% of the maximum at 7 days (Roby et al., 1991). Tomato plants infected with *Cladosporium fulvum* steadily accumulated in apoplastic chitinases over 10 days, after which the levels plateaued (Joost et al., 1989).

The pathogenic induction of chitinase activity could result from the induction of chitinase gene expression or by increasing the stability of chitinase mRNA, but it is not yet known which is the case. Chitinase mRNA accumulated locally in the leaves of tobacco infected with tobacco mosaic virus (TMV) (Brederode et al., 1991).

As stated before, both pathogenicity and compatibility influence the strength of the induction of chitinase activity. A compatible interaction between pathogen and plant results in multiplication of the pathogen and ultimately the spread of the disease. An incompatible interaction between pathogen and plant results in a hypersensitive response in which necrotic lesions occur at the infection sites. Chitinases have been shown to be involved in hypersensitive responses. Fink et al. (1990) showed that oat infected with

compatible *Puccinia* did not produce chitinase, but incompatible *Puccinia* infections rapidly induced chitinase activity. Voisey and Slusarenko (1989) infected the leaves of French bean with virulent and avirulent races of *Pseudomonas syringae* pv. *Phaseolicola* and found that the avirulent pathogens caused an incompatible infection in the affected leaf. Chitinase activity in the infected leaf increased within 6 to 9 hours and rose 19-fold when compared to uninfected leaves after 48 hours. However, chitinase activity in the bean leaf infected by the virulent race did not increase until 24 hours and by 48 hours rose to only 75% of the resistant reaction. Daurois et al. (1990) found that accumulation of induced chitinase occurs earlier in resistant cultivars than in susceptible ones. However, chitinase and chitinase mRNA levels in susceptible plants never achieved the same levels as in the resistant plants. This indicates that the induction of chitinase can occur regardless of whether the plant was resistant or susceptible. The major difference is that the induction of chitinase expression in susceptible plants may be retarded relative to resistant plants. The induction of chitinase expression does not depend on the presence of living infectious organisms. It was found that both heat-killed virulent and avirulent *Pseudomonas syringae* cells induced chitinase activity in French bean leaves (Voisey and Slusarenko, 1989). It is possible that components of pathogen cell walls rather than metabolites released from a metabolically active cell are responsible for chitinase induction. Additionally, infection could induce the expression of chitinase and  $\beta$ -1, 3 glucanase activities. Pathogenic infection causes the coordinate induction of chitinase and  $\beta$ -1, 3-glucanase activities in tobacco, maize, and tomato (Meins and Ahl, 1989;

Cordero et al., 1994; Joosten et al., 1989). Interestingly, colonization of soybean roots by *Bradyrhizobium japonicum* also induced the expression of chitinase (Stahelin et al., 1992). The induction did not occur in the central region of effective nodules but in cortex in both effective and ineffective nodules. Another symbiotic organism, *Glomus versiforme*, also induces chitinase activity in leek (*Allium porrum*) (Spanu et al., 1989). Finally, infection does not always elevate chitinase expression. Godiard et al. (1990) found that the accumulation of a chitinase mRNA did not increase chitinase in tobacco leaves infected with compatible, incompatible, or avirulent strains of *Pseudomonas solanacearum*.

### **Molecular cloning of plant chitinase genes**

Chitinase genes and cDNA clones for chitinases have been isolated from many plant species, especially dicots. The general strategy for cloning genes encoding plant chitinases is via genomic DNA library or cDNA library screening with a homologous or heterologous probe, while the use of PCR-based cloning techniques has increased recently. Cloning of genes or cDNAs for chitinases has been reported for *Arabidopsis* (Samac et al., 1990), bean (Broglie et al., 1986), barley (Swegle et al., 1989), cucumber (Lawton et al., 1994), garlic (Van Damme et al., 1993), potato (Laflamme and Roxby, 1989), peanut (Herget et al., 1990), rapeseed (Rasmussen et al., 1992a), rice (Huang et al., 1991), and sugar beet (Nielsen et al., 1993). Promoters for specific chitinase genes have also been characterized (Roby et al., 1990; Ficker et al., 1997).

Many plant chitinases are encoded by a small multigene family. In tobacco, seven distinct clones for class I chitinases have been recovered (Fukuda et al., 1991; Hooft van Huidsdijnen et al., 1987; Neale et al., 1990; Shinshi et al., 1987; 1990). However, in *Arabidopsis*, both basic and acidic chitinases are encoded by a single-copy gene (Samac et al., 1990). Usually, the length of the coding region for plant chitinases is about 1kb. For example, the genes for class I chitinases are relative small, requiring less than 2kb for the open reading frame, which may or may not be interrupted by an intervening sequence. The *Arabidopsis* class I chitinase gene has one intron (Samac et al., 1990), while tobacco CHN14, CHN48, and CHN50 chitinase genes have two introns (Shinshi et al., 1990; Van Buren et al., 1992). Bean CH5B (Broglie et al., 1989) and rice RCH10 chitinase genes (Zhu and Lamb, 1991) have no introns.

### **Roles of chitinases in plants**

*In vitro* studies have shown that chitinases have an inhibitory effect upon the growth of chitin-containing fungi. Most of the effective chitinases are class I (Broekaert et al., 1988; Mauch et al., 1988b; Roberts and Selitrennikoff, 1988; Schlunbaum et al., 1986). Chitin and  $\beta$ -1, 3-glucan fibers are synthesized simultaneously in the apex of the growing hyphae during apical growth in filamentous fungi. In the mature cell wall distant from the apex, the polysaccharides are cross-linked to form mixed chitin-glucan fibers and may be overlaid by other polysaccharides and protein layers (Wessels, 1986; 1988). Thus, at the apex, the exposed nascent chitin chains are accessible to hydrolysis by chitinase, whereas the chitin layer in the mature cell wall is inaccessible to

degradation by the enzyme. This theory is supported by an autoradiographic study on *Cercospora beticola* showing that a purified sugar beet chitinase was able to degrade newly synthesized radioactively labeled chitin fibers in the hyphal apex, whereas it had no effect on the chitin layer in the mature cell wall (Nielsen and Mikkelsen, 1994). It was also found that chitinases caused apical swelling and /or lysis of immature fungal cell walls (Boller et al., 1983; Brokaert et al., 1988; Mauch et al., 1988b; Ordentlich et al., 1988).

Direct evidence that the enzyme interacts with fungal cell walls *in vivo* comes from immunocytochemical techniques (Benhamou et al., 1990; Wubben et al., 1992). It was demonstrated that the enzyme accumulated around fungal cell walls *in planta*. Chitinase was mainly associated with altered fungal cell walls, suggesting that the enzyme activation is either preceded by the hydrolytic action of other enzymes such as  $\beta$ -1, 3-glucanase or coincides with these enzymes. With respect to their localization and implication in the defense reaction, it has been proposed that extracellular chitinases are part of an early, induced response. These extracellular chitinases may act directly by blocking the growth of the hyphae invading the intercellular space. Perhaps chitinases act indirectly as well by releasing fungal elicitors that induce additional chitinase activity and other defense reactions in the host (Barber et al., 1989; Kurosaki et al., 1988; Roby et al., 1987). Large quantities of vacuolar chitinase may be rapidly released, which would overwhelm the natural balance of fungal cell wall construction at the hyphal tip and then encourage the osmotic lysis of the hyphae (Keefe et al., 1990; Mauch and Staehelin,

1989). The release of vacuolar chitinase would preclude long-term adaptation of the fungal pathogen to higher chitinase concentrations as might occur if the plant expressed constitutively high chitinase levels.

As stated before, the expression of chitinase is regulated in a developmental and organ-specific manner. High expression of chitinase in roots may indicate that the enzyme kills or slows the growth of soil fungi and bacteria that constantly attack the plant, making the fungal colonization of roots more difficult.

Chitinases are found in many seeds such as maize (Huynh et al., 1992), barley (Jacobsen et al., 1990), wheat (Molano et al., 1979), rice (Nishizawa et al., 1991), rye (Yamagami et al., 1994), and soybean (Yeboah et al., 1998). Since seeds are responsible for the success of the future generation, it is reasonable to hypothesize that seeds should require greater antifungal protection than the rest of the plant.

Not all chitinases show antifungal properties. In tobacco, class I chitinases are strongly antifungal, particularly in combination with class I  $\beta$ -1, 3-glucanases. However, class II chitinases from tobacco demonstrate no antifungal activity alone and only moderate activity when in combination with class I  $\beta$ -1, 3-glucanases (Sela-Burlage et al., 1993). The reason may be that class I chitinases have a greater probability of successfully attacking the fungal cell wall because they contain a chitin-binding domain, but this is not yet clear.

Although much attention has been devoted to the study of the antifungal function of chitinase, other potential functions of plant chitinase in plant growth and development have received comparatively little attention. One recent study showed that a temperature-sensitive development carrot mutant could only proceed past the globular stage at the nonpermissive temperature when an acidic endochitinase was provided (De Jong et al., 1992). Chitinases from barley, tomato, and sugar beet could not replace this chitinase. These studies indicate that some chitinases may have endogenous functions as well as antifungal functions.

Chitinases can be induced in plants by symbiotic bacteria. Recent studies have shown that chitinase might be involved in nodule development (Staehelin et al., 1992; Vasse et al., 1993; Parniske et al., 1994; Goormachtig et al., 1998; Xie et al., 1999). More directly, rhizobial Nod factors are substrates for plant chitinases (Staehelin et al., 1994a; 1994b; Goormachtig et al., 1998; Minic et al., 1998; Schultze et al., 1998). Nod factors are rhizobial lipo-chitooligosaccharide signals that trigger root nodule development in legumes. Specific chitinase isoforms exhibit distinct substrate specificity and cleavage site preference toward Nod factors. For example, the sulfate group at the reducing end protects tetrameric Nod factors in *Sinorhizobium meliloti* against hydrolysis by various chitinases (Staehelin et al., 1994b; Schultze et al., 1998). It was also found that fucosylation of Nod factors of *Rhizobium leguminosarum* bv. *Viciae* conferred protection against hydrolysis by certain plant chitinases *in vitro* (Ovtsyna et al., 2000). Degradation products, such as lipo-disaccharides and lipo-trisaccharides, showed strongly

reduced biological activity on host plants. This indicates that plant chitinases can inactivate Nod factors. Expression of a chitinase gene from *Serratia marcescens* in *Sinorhizobium* strains impeded nodulation (Krishnan et al., 1999).

### **Creation of transgenic plants to investigate the role of chitinase**

The activity of chitinases against fungi makes this protein an attractive candidate to increase pest resistance of crop plants. Transgenic techniques have been widely used for gene expression studies and genetic engineering of disease resistant plants. With its potential antifungal properties, it is possible to constitutively over-express a chitinase gene in transgenic plants resulting in enhanced resistance to one or more fungal pathogens. The first report of transferring a chitinase gene to foreign plants was performed with tobacco and *Brassica napus*. A bean vacuolar chitinase gene under the control of the strong constitutive promoter from the cauliflower mosaic virus (CaMV) 35S was transferred to tobacco and *Brassica napus*. Transgenic plants showed decreased symptoms of post emergence damping-off causing by *Rhizoctonia solani*, (Broglie et al., 1991). Significant reduction in fungal growth and delay in disease development was observed in transgenic lines. A rice chitinase gene, RCC2, was transferred to cucumber and *Chrysanthemum* and the transgenic plants showed enhanced resistance to gray mold (*Botrytis cinerea*) when compared to non-transgenic control plants (Tabei et al., 1998; Takatsu et al., 1999). In transgenic cucumber plants with the highest resistance against *B. cinerea*, the spread of disease was inhibited completely. Transgenic *Chrysanthemum*

plants with greater resistance to *B. cinerea* showed very slight symptoms, and the symptoms did not spread when the incubation period was extended.

Chitinase genes cloned from bacteria, fungi and insects also have been transferred to plants. Transgenic tobacco over-expressing two bacterial chitinase genes at high levels demonstrated that the degree of resistance to *Rhizoctonia solani* increased with increased chitinase gene expression. The reduction in disease symptoms obtained was almost 40% (Dunsmuir et al., 1993). Studies on transgenic tobacco and potato expressing a gene encoding a strongly antifungal endochitinase from a fungus showed that the transgenic plants were highly tolerant or completely resistant to the foliar pathogens *Alternaria alternata*, *A. solani*, *Botrytis cinerea* and the stillborn pathogen *Rhizoctonia solani* (Lorito et al., 1998). Moreover, transgenic tobacco expressing an insect chitinase gene showed improved insect resistance (Ding et al., 1998). The growth of tobacco budworm (*Heliothis virescens*) larvae was reduced after feeding on the transgenic plants. The feeding damage the budworm caused on the transgenic plants was also reduced. This indicates that plants expressing an insect chitinase gene may have agronomic potential for insect control.

Instead of using a constitutive promoter such as 35S to ensure the expression of chitinase genes in the transgenic plants, it is also possible to use tissue specific promoter to direct the location of the chitinase gene expression. A promoter for potato pistil-specific basic endochitinase has been cloned and has effectively directed the expression

of GUS to the pistil (Ficker et al., 1997). Since roots are the organ of the plant that are most subject to pathogen attack, it would be very beneficial to over-express chitinase specifically in roots via a root-specific promoter, although none has yet been cloned.

Not all transgenic plants have shown enhanced disease resistance. Transgenic tobacco constitutively expressing a tobacco chitinase gene accumulated up to 120-fold more active chitinase than non-transformed plants, but did not exhibit increased resistance to the fungus *Cercospora nicotiana* (Neuhaus et al., 1991b). This chitinase gene may not be involved in plant resistance to this particular fungus, and other chitinase genes may have a more dramatic effect. It is also possible that over expression of only chitinases may be insufficient to provide enhanced disease resistance, and co-expression of both chitinase and  $\beta$ -1, 3 glucanase may provide a better protection against a wide range of pathogens. Co-expression of both a rice basic chitinase gene and an alfalfa acidic  $\beta$ -1, 3 glucanase gene showed substantially greater protection against the fungus *Cercospora nicotiana* than transgenic tobacco expressing either gene alone (Zhu et al., 1994). Similar results were also found in transgenic tomato plants (Melchers et al., 1993).

Nevertheless, transgenic plants harboring chitinase genes have shown increased resistance against several fungal pathogens, and this may prove to be a practical approach for controlling some crop fungal diseases. Future improvements in the chitinase-mediated increase in resistance may include the production of plant lines

modified with several pathogen resistance genes, selection of pathogen-tolerant plant lines for further modification, and careful selection of the chitinase gene used.

## INTRODUCTION

Chitin, an insoluble linear polymer of  $\beta$ -1, 4-linked N-acetylglucosamine (NAG), is one of the most abundant polysaccharides in nature. Chitin has been found to be a constituent of insect exoskeletons, crustacean shells, and fungal cell walls. Chitinase is an enzyme that catalyses the hydrolysis of a bond between the C1 and C4 of two consecutive NAGs of chitin. Chitinase is found in a wide range of organisms including bacteria (Roberts et al., 1982), fungi (Ohtakara et al., 1961), insects (Koga et al., 1983), crustaceans (Lunt et al., 1960), and many higher plants (for reviews, see Collinge et al., 1993; Graham et al., 1994). A major role of chitinases in fungi, insects and crustaceans is the modification of the organism's structural constituent chitin. Bacteria produce chitinase to digest chitin and utilize it as a carbon and energy source.

Chitinases are expressed constitutively at low levels in some plants (Beerhues et al., 1990), and activity can be induced dramatically by wounding or by infection of the tissue with fungal or bacterial pathogens (Majeau et al., 1990; Roby et al., 1990). Many plant chitinases can also be induced during pathogenic attack (Herget et al., 1990) or environmental stresses (Van Damme et al., 1993). Chitinases inhibit fungal growth either *in vitro* (Schlumbaum et al., 1986; Leah et al., 1991) or when expressed in transgenic plants (Broglie et al., 1991; Jach et al., 1995). Since no endogenous substrate has thus

been found for chitinase in higher plants, it has been postulated that chitinase may play a role in plant defense mechanisms.

Chitinases have been studied extensively in mature higher plants and in pathogen-infected plants (for a review, see Graham et al., 1994). However, biochemical and molecular analysis of chitinases from seed sources are still relatively limited, with most characterization reported for a few monocot cereal grains (Huynh et al., 1992; Jacobsen et al., 1990; Leah et al., 1991; Molano et al., 1979; Nishizawa et al., 1991; Yamagami et al., 1994; Krishnaveni et al., 1999; Caruso et al., 1999). A few chitinase characterizations have been reported for dicots including soybean and kidney bean (Yeboah et al., 1998; Ramos et al., 1998; Gomes et al., 1996). In general, all plant chitinases are small proteins with molecular weights ranging from 25,000 to 40,000 kD with a pH optimum around 5. Plant chitinases typically exist as monomers and are either acidic or basic because of their low or high isoelectric points, respectively.

For many important crop species, seeds are the vital link between subsequent generations. In the field, seeds germinate in an environment rich in microorganisms, both beneficial and pathogenic. Hence, in addition to the hydrolases essential for nutrient reserve mobilization involved in expansive growth and degradation of barrier tissues surrounding the embryo, seeds would be predicted to have highly evolved defense mechanisms to give rise to healthy seedlings.

A seed may protect itself from pathogenic attack by having physical barriers such as a hard coat. However, some pathogens have the ability to penetrate the seed coat and infect seeds, particularly at the time of radicle emergence when physical barriers are broken and the embryo is fully exposed and vulnerable. So other defense mechanisms, such as non-enzymatic biochemical compounds or hydrolytic enzymes, might exist in seeds in combination with physical barriers to provide a battery of protective mechanisms (Flach et al., 1992). It has long been hypothesized that chitinases present in seeds may help protect them against chitin-containing parasites such as fungi (Powning and Irzykiewicz, 1965). Chitinases have been found in dry seeds (Krishnaveni et al., 1999; Ramos et al., 1998). Chitinases are also found to be selectively released into the surrounding medium during the early stages of imbibition by barley seeds (Swegle et al., 1992). Some chitinases are also induced in response to fungal infection during germination of maize and wheat seeds (Cordero et al., 1994; Caruso et al., 1999). Chitinases isolated from cowpea seeds apparently have the ability to inhibit the growth of *Colletotrichum lindemuthianum* and *Colletotrichum musae* and to negatively affect the development of the cowpea weevil (*Callosobruchus maculatus*) in an artificial seed system (Gomes et al., 1996).

Muskmelon is an important crop in America. High-yielding production depends on a good seedling establishment. Although muskmelon seeds germinate well at a wide range of temperatures, they are also very susceptible to pathogen attack, particularly by *Pythium*. The long-term goal of research in Dr. Gregory Welbaum's lab is to investigate

how muskmelon seeds protect themselves during seed development, imbibition or after radicle emergence. The questions that I will address in this dissertation are:

1. Does chitinase activity exist in muskmelon seeds?
2. What genes encode chitinases expressed in muskmelon seeds?
3. Under what conditions are chitinase genes expressed in muskmelon seeds?
4. How is chitinase gene expression regulated in muskmelon seeds?

## CHAPTER TWO

### CHARACTERIZATION OF CHITINASE ACTIVITY IN MUSKMELON SEEDS

#### Materials and Methods

##### Plant Material

Muskmelon (*Cucumis melo* cv. Top Mark) seeds were decoated and germinated at 30°C in the dark on Petri dishes with three layers of germination blotter paper (Anchor Paper Co.) moistened with distilled water. In some cases, decoated seeds were dissected to separate the endosperms from embryonic tissues. In other cases, seeds were dissected to yield the embryonic axis, cotyledons, the endosperm tip adjacent to the embryonic axis, and the remaining endosperm tissue.

##### Protein Extraction

Crude protein was extracted from dry seeds, germinating and germinated muskmelon seeds, or isolated seed tissues by homogenizing in 50 mM potassium phosphate buffer, pH 6.8 at a ratio of 1:15 (tissue: buffer, w/v) with an ice-chilled mortar

and pestle. The homogenate was clarified by centrifugation at 10,000g for 5 min, and the supernatant was assayed for enzyme activity.

#### Gel Diffusion Assay for Chitinase Activity

A gel-diffusion assay, similar to those used for other hydrolases (Dingle et al., 1953; Wood et al., 1988; Downie et al., 1994), was developed to evaluate chitinase activity. Briefly, the assay is based upon diffusion of the enzyme from a central well through agarose gel containing the substrate glycol chitin, a soluble modified form of chitin, previously used for assaying chitinase activity (Trudel et al., 1989; Pan et al., 1991). Glycol chitin was synthesized as described by Trudel and Asselin (1989). Chitinase diffuses from the well and catalyzes the cleavage of glycol chitin leaving a clear non-fluorescent zone in the gel, the diameter of which is proportional to enzyme activity. The detection of chitinase activity is based on the affinity of calcofluor for chitin (Maeda et al., 1967). Calcofluor when bound to undigested chitin fluoresces under UV light. The region where glycol chitin was digested by chitinase appears dark under UV light, as calcofluor does not have affinity for digested chitin.

Gel plates for chitinase assays were prepared by melting 1.6 % (w/v) agar in incubation buffer (0.1M citric acid, 0.2 M sodium phosphate, pH 5.0). The solution was cooled to 50 to 60 °C and glycol chitin (0.5%) was added to the solution and mixed well. 30 mL solution was dispensed into a disposable 140- x 15-mm Petri dish (Falcon, Becton

Dickinson). When the solution had solidified, wells were punched with a cut-off pipette tip, and cores were removed.

10  $\mu\text{L}$  crude protein extracts from seeds or seed tissues or 10  $\mu\text{L}$  standard chitinase enzyme from *Serratia marcescens* (Sigma) with concentrations of  $5 \times 10^{-5}$  unit  $\mu\text{L}^{-1}$ ,  $1 \times 10^{-4}$  unit  $\mu\text{L}^{-1}$ ,  $3 \times 10^{-4}$  unit  $\mu\text{L}^{-1}$ ,  $5 \times 10^{-4}$  unit  $\mu\text{L}^{-1}$ , or  $1 \times 10^{-3}$  unit  $\mu\text{L}^{-1}$ , 10  $\mu\text{L}$  incubation buffer, and boiled crude protein extracts were added to the wells. After incubation at 28 °C for 20 hours, the gel was stained with 20 mL 0.1% calcofluor (dissolved in 0.5M Tris-HCl, pH 8.9) for 10 min. The gel plate was washed with distilled water, and chitinase activity was visualized under a UV light.

#### Native-PAGE and Activity Staining for Chitinases

Native –PAGE for chitinase was performed in 7.5% (w/v) polyacrylamide gel containing 0.5% glycol chitin as the substrate. 20  $\mu\text{L}$  crude protein extracts were subjected to electrophoresis at either pH 8.8 for acidic chitinases or pH 4.3 for basic chitinases. After electrophoresis, the gel was equilibrated in 0.1M citric acid / 0.2 M sodium phosphate buffer (pH 5.0) at 28 °C for 20 h and then stained with 0.1% calcofluor. After washing the gel with distilled water, the activity of chitinase isoforms was visualized under UV light.

#### Antifungal Inhibition Assay

Antifungal inhibition assay was done following the method described by Mauch et al. (1988). All manipulations were carried out under sterile conditions. *Pythium*

*ultimum*, *Rhizoctonia solani*, *Phytophthora infestans*, and *T. viride* were grown on the centers of Petri dishes containing the nutrient agar used for maintenance of the test fungus. Sterile filter paper discs were laid on the agar surface, and 20  $\mu\text{L}$  crude protein extracts isolated from seeds with imbibition times of 8, 16 or 24 h, water, and standard chitinase enzyme ( $3 \times 10^{-4}$  unit  $\mu\text{L}^{-1}$ , from *Serratia marcescens*) were applied to the discs. All solutions were filtered through a 0.22  $\mu\text{M}$  membrane filter prior to application. The plates were further incubated at room temperature. Antifungal activity was checked afterwards.

## **Results**

As a first step toward characterizing chitinase activity in muskmelon seeds, a gel diffusion assay was developed. Chitinase activity was detected in muskmelon seeds during imbibition and after radicle emergence (radicle starts to emerge at about 16 h after the start of imbibition at 30°C) (Fig. 2-1). Chitinase activity was also detected in mature dry seeds (data not shown here). When muskmelon seeds were dissected and separated into endosperm and embryo tissues, chitinase activity was detected in both tissues. Interestingly, on a fresh weight basis, chitinase activity in the endosperm tissue was much higher than in embryo tissue. Standard enzyme purified from *Serratia marcescens* was used as a positive control. As indicated by the standard enzyme, the diffusion zone area

was proportional to units of the enzyme applied. Neither the incubation buffer nor boiled crude protein extracts showed enzymatic activity.

By assaying muskmelon seeds at 20, 30, 40, and 50 days after anthesis (DAA) as well as fresh seeds from mature edible fruit, it was found that chitinase activity was detectable in seeds by 40 DAA (Fig. 2-2). Chitinase activity increased with maturation and was correlated with the onset of seed germinability that began at 35 DAA. Chitinase activity detected in imbibed seeds or in seeds after radicle emergence (Fig. 2-1) was higher than that in young developing seeds (Fig. 2-2).

Chitinases have been described as acidic or basic based upon their isoelectric points. Native-PAGE and activity staining was used to identify different chitinase isoforms in muskmelon seeds. One acidic chitinase isoform, AD1, was detected in young muskmelon seeds at least 40 DAA, and its activity increased with further maturation (Fig. 2-3). No acidic chitinase isoform was detected in very young seeds at 20 and 30 DAA. These results are consistent with those from the gel diffusion assay in Fig. 2-2.

Basic chitinase isoforms were also detected in young developing and fresh mature muskmelon seeds. Three basic chitinase isoforms were detected in seeds that were at least 40 DAA (Fig. 2-4). BD3 basic isoform activity was greater than that of BD1 or BD2 basic isoform. No basic chitinase isoforms were detected in very young seeds at 20 and 30 DAA.

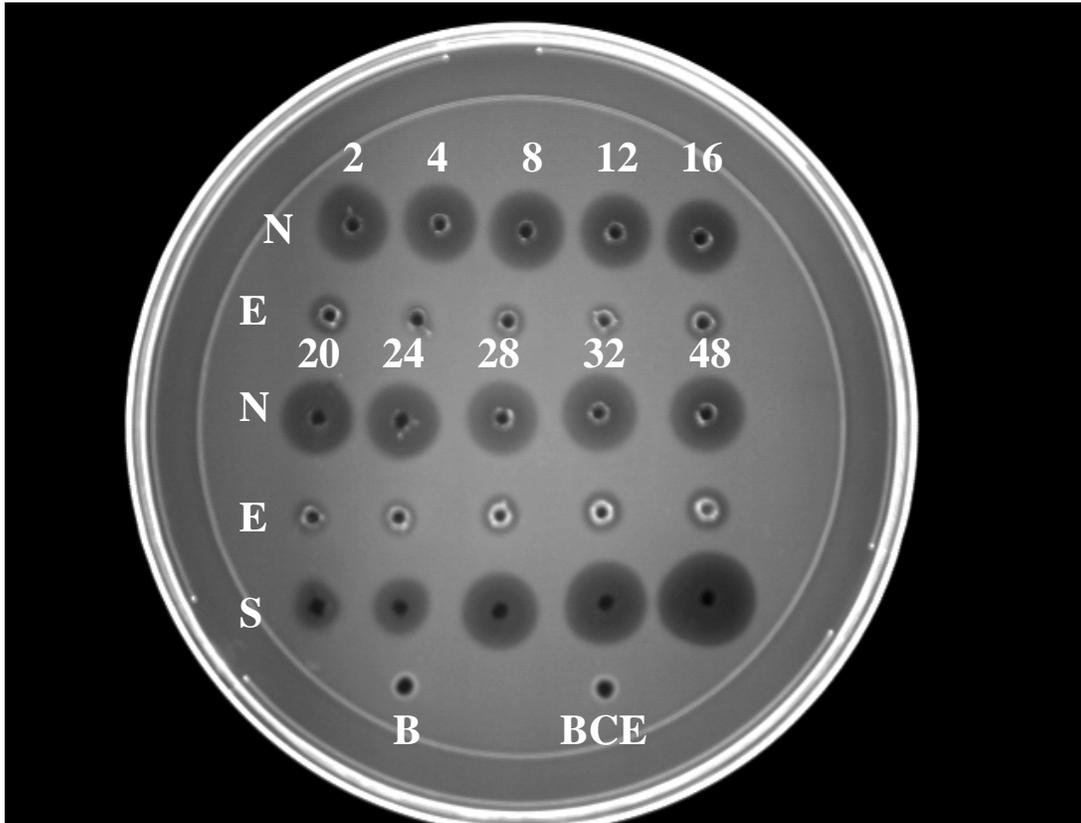
To determine timing and tissue localization of different chitinase isoforms, muskmelon seeds were imbibed for 2, 4, 8, 12, 16, 20, 24, 28, 32, or 48 h and dissected into endosperm and embryo tissues. Activity gel staining revealed that one acidic chitinase isoforms were in endosperm tissues from imbibed seeds and seeds after radicle emergence (Fig. 2-5). One major acidic isoform AE1 was expressed in the endosperm tissue during imbibition and after radicle emergence. Another acidic chitinase isoform AE2 was detected only in seeds after 16 h imbibition time. At 30°C, 16 h is the initial time of radicle emergence for some muskmelon seeds. Activity of AE2 isoform increased after radicle emergence. Basic chitinase isoforms also exist in the endosperm tissue during seed imbibition and after radicle emergence (Fig. 2-6). No acidic chitinase was detected early in embryo tissues isolated from imbibed seeds (Fig. 2-7). Some acidic chitinase activity was detected in seeds after radicle emergence

To confirm the results of the experiment described in Figure 2-5 and 2-7, a second related experiment was performed. Muskmelon seeds imbibed for 12 h were dissected into embryonic axis (EA), cotyledon (C), endosperm tip adjacent to the embryonic axis (NT), and remaining endosperm tissue (N). Crude protein was isolated from each tissue and subjected to native-PAGE. As shown in Figure 2-5 and 2-7, an acidic chitinase AE1 isoform was abundant in the endosperm tissue (Fig. 2-8). Within the endosperm tissue, higher activity was detected in the remaining endosperm tissue than in the endosperm tip adjacent to the embryonic axis. No acidic isoform was detected in the embryonic axis or cotyledon tissues.

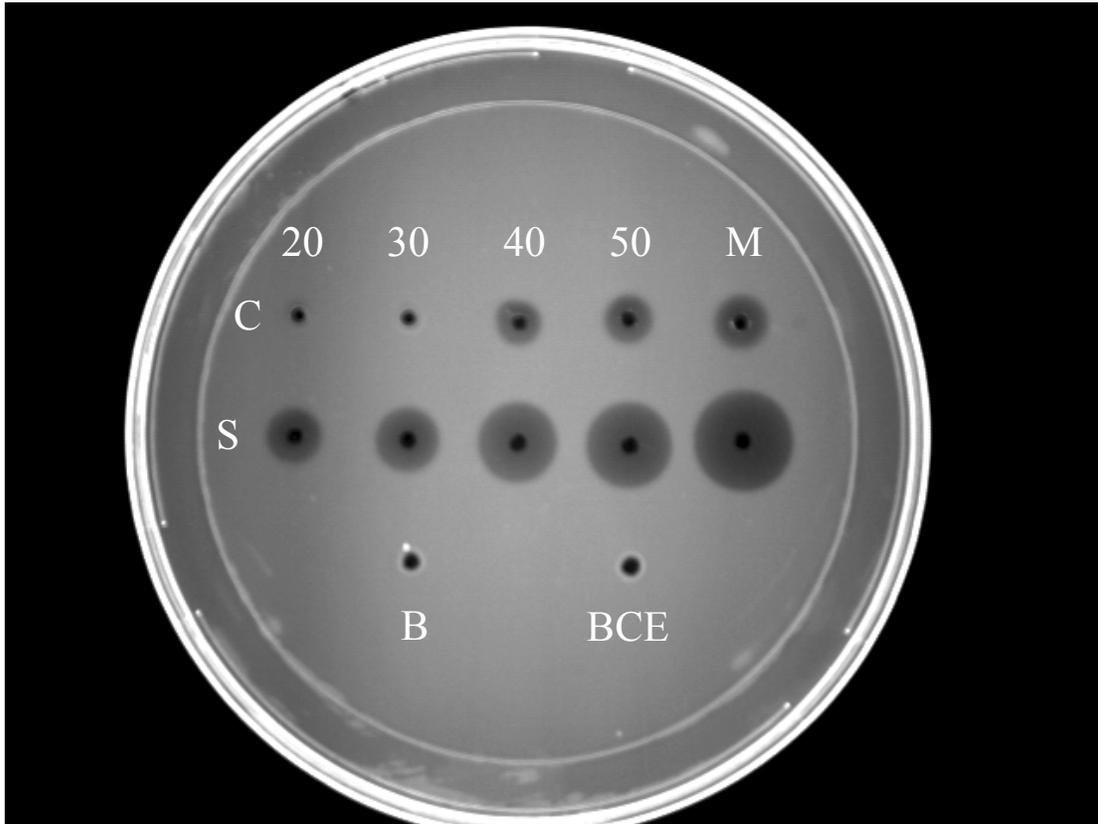
Although no acidic isoform was detected in embryo tissue, three basic chitinase isoforms were found in embryo tissue isolated from freshly imbibed seeds or in seeds after radicle emergence (Fig. 2-9). Two major isoforms, BE1 and BE2, were detected in the embryo tissue. The activity of the BE3 isoform was barely detectable on the gel. Three basic chitinase isoforms were detected in embryonic axis tissue isolated from imbibed seeds or radicles isolated from seeds after radicle emergence (Fig. 2-10). Activity of the BR3 basic isoform increased after radicle emergence while activity of the BR1 and BR2 basic isoform decreased after radicle emergence.

Crude protein extracts were isolated from imbibed seeds with imbibition times of 8, 16, or 24 h. To test for antifungal activity, aliquots of the sterile filtered proteins (20  $\mu$ L) were added to filter paper discs on agar plates containing fungus. No inhibition of fungal growth was observed for any sample checked, including standard enzymes.

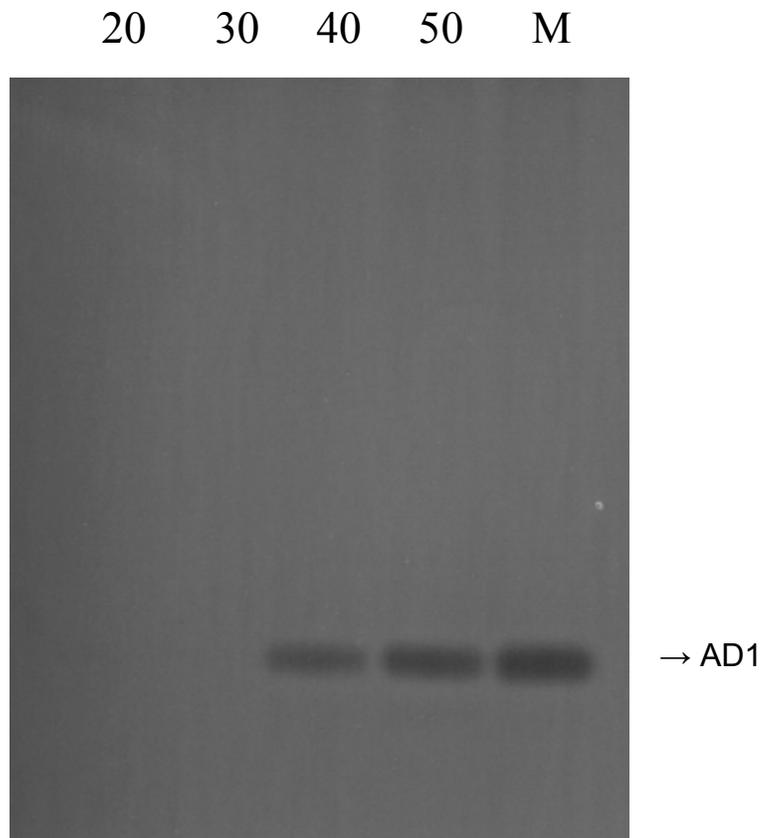
## **Figures**



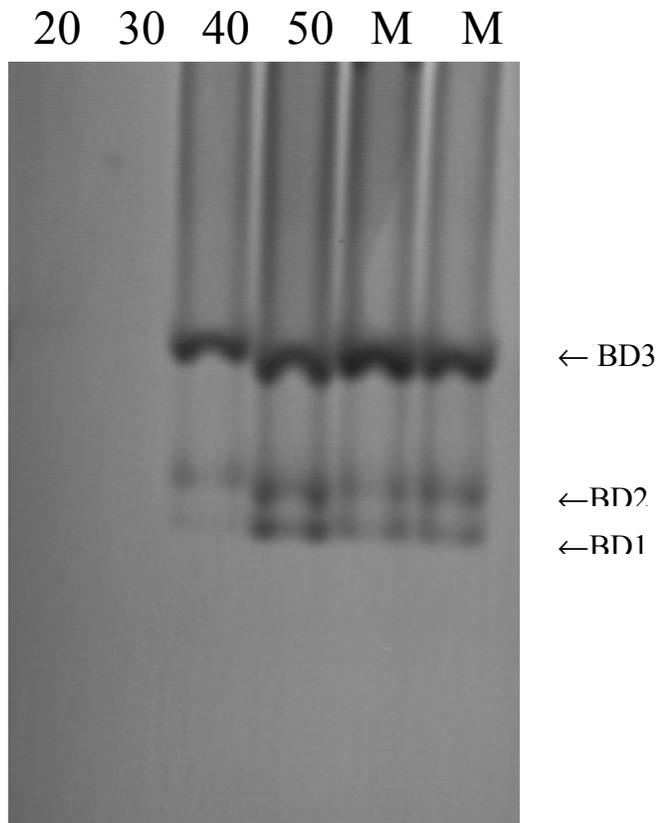
**Figure 2-1. Gel diffusion assay of chitinase activity from endosperm and embryo from muskmelon seeds.** Endosperm (N) and embryo (E) were isolated from decoated muskmelon seeds imbibed in water for 2, 4, 8, 12, 16, 20, 24, 28, 32 or 48 h. Each well in row labeled N and E contained 10  $\mu$ L crude protein extract. Row S contained serial dilutions of purified standard chitinase from *Serratia marcescens*. Well B contained incubation buffer (0.1 M citric acid/ 0.2 M sodium phosphate) and well BCE contained boiled crude extracts (BCE).



**Figure 2-2. Gel diffusion assay of chitinase activity in developing and mature muskmelon seeds.** Row C contained 10  $\mu$ L crude protein extract from developing muskmelon seeds at 20, 30, 40, or 50 days after anthesis (DAA) and from mature seeds (M). Row S contained dilutions of standard chitinase as in Fig. 2-1. Control B and BCE were described as in Fig. 2-1.

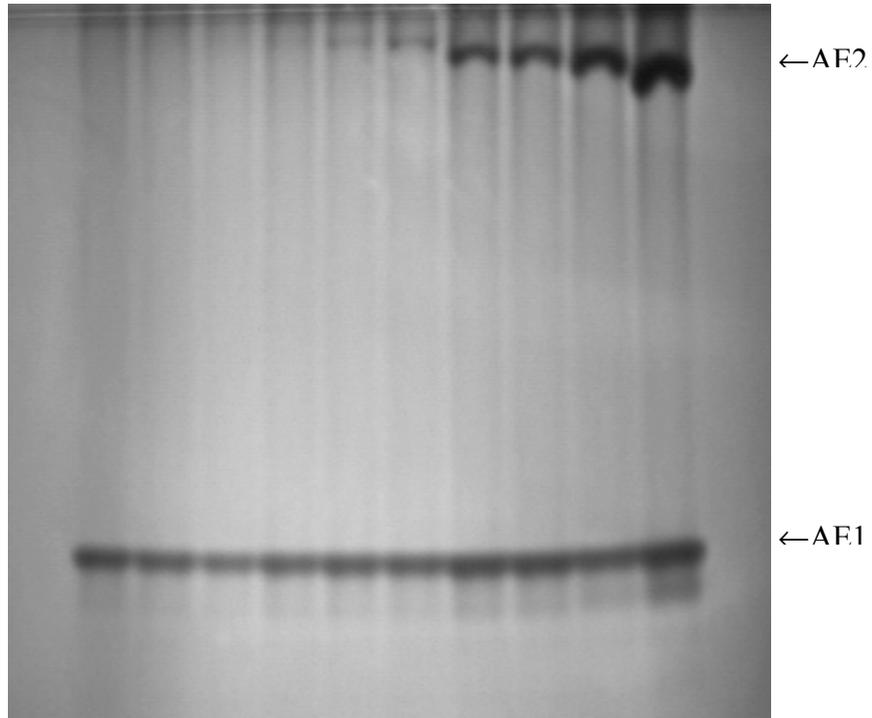


**Figure 2-3. Native-PAGE and activity staining of acidic chitinase isoform from muskmelon seeds at different developmental stages.** Crude protein extracts were prepared from seeds at 20, 30, 40, or 50 DAA and from mature seeds (M). For each lane, 20  $\mu$ L crude extract were subjected to electrophoresis at pH 8.8 through a 7.5 % polyacrylamide gel containing 0.5% glycol chitin. Detection of chitinase activity was as described in Materials and Methods.

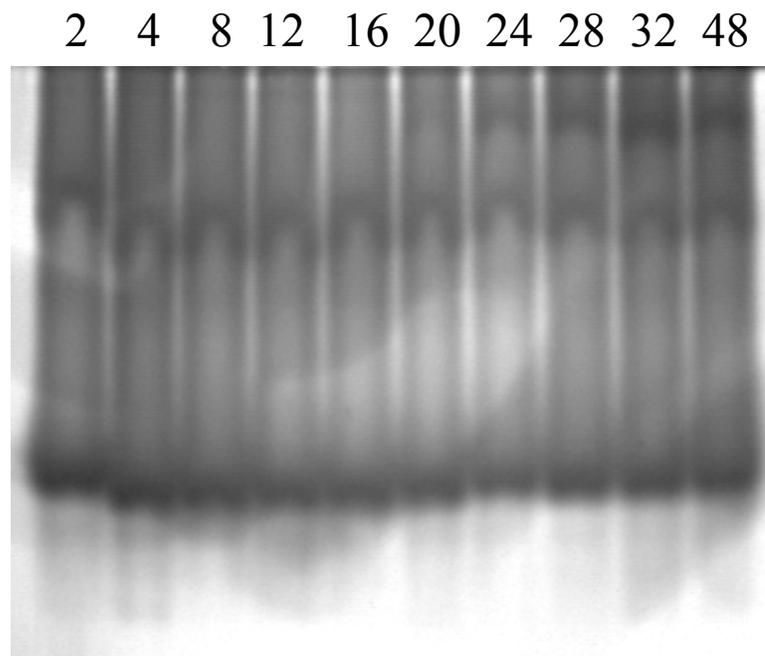


**Figure 2-4. Native-PAGE and activity staining for basic chitinase isoforms from muskmelon seeds at different developmental stages.** Crude protein extracts were prepared from muskmelon seeds at 20, 30, 40, or 50 DAA and from mature seeds (M). For each lane, 20  $\mu$ L crude protein extract were subjected to electrophoresis at pH 4.3 through a 7.5% polyacrylamide gel containing 0.5% glycol chitin. Detection of basic isoforms BD1, BD2 and BD3 was as described in Materials and Methods.

2 4 8 12 16 20 24 28 32 48



**Figure 2-5. Native-PAGE and activity staining for acidic chitinase isoform from endosperm tissue.** Crude protein extracts were isolated from endosperm tissues of seeds imbibed for 2, 4, 8, 12, 16, 20, 24, 28, 32, or 48 h. 20  $\mu$ L crude protein from each sample were subjected to electrophoresis as described in Fig. 2-3.

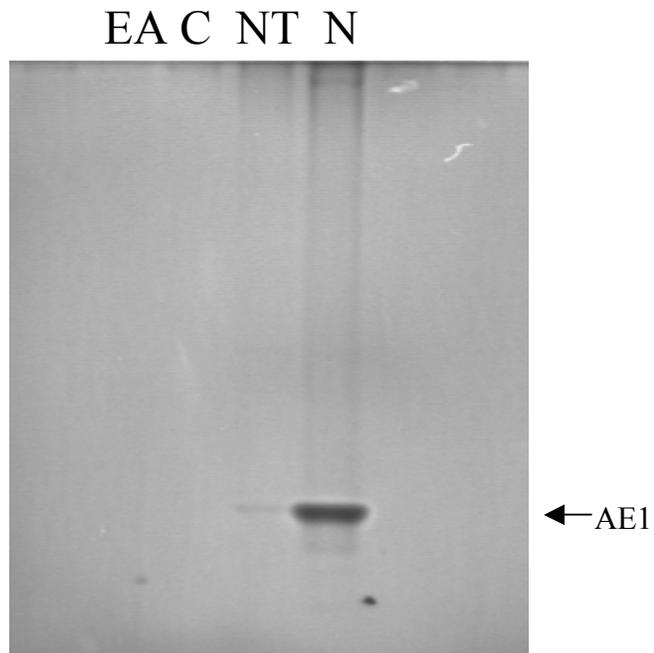


**Figure 2-6. Native-PAGE and activity staining for basic chitinase from endosperm tissue.** Crude protein extracts were isolated from endosperm tissues of seeds imbibed for 2, 4, 8, 12, 16, 20, 24, 28, 32, or 48 h. 20  $\mu$ L crude protein from each sample were subjected to electrophoresis as described in Fig. 2-4.

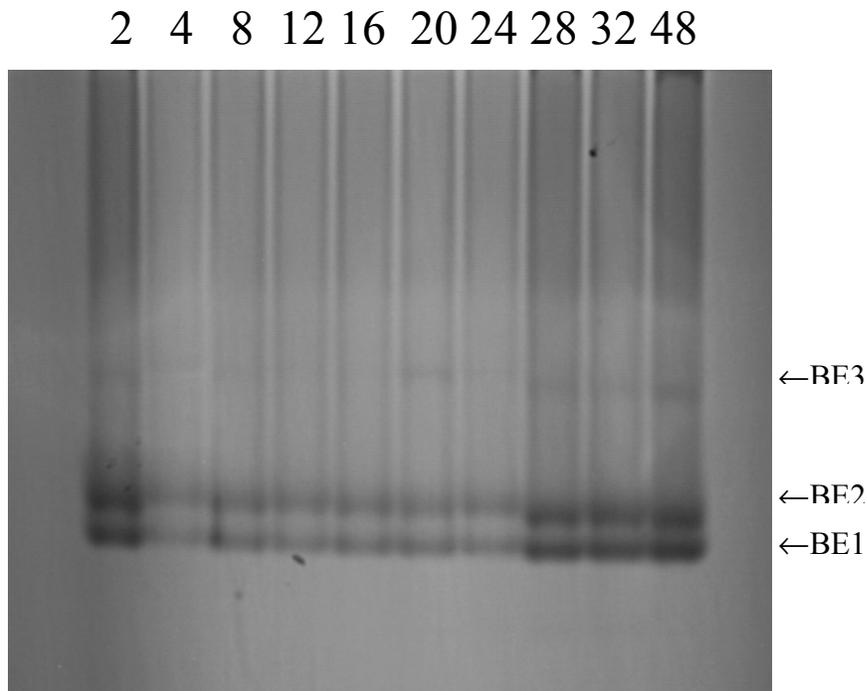
2 4 8 12 16 20 24 28 32 48



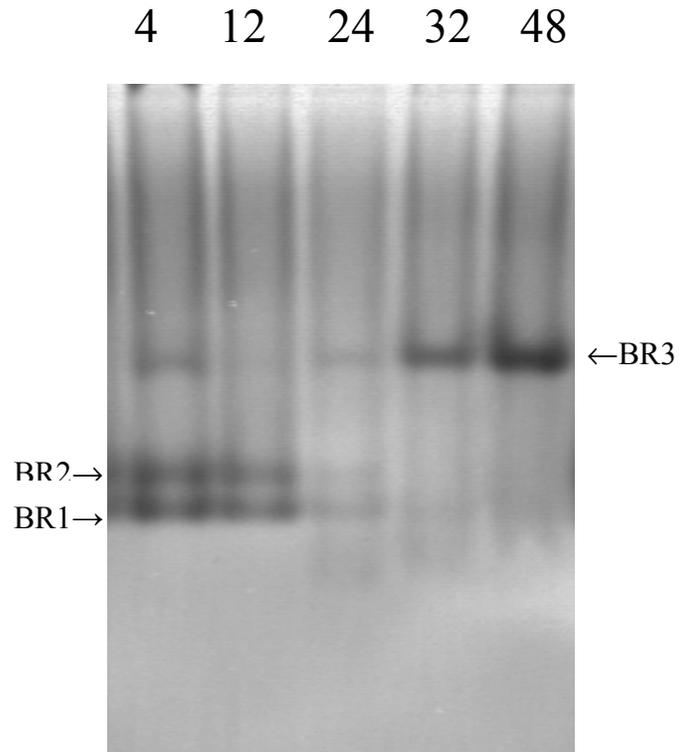
**Figure 2-7. Native-PAGE and activity staining for acidic chitinase from embryo tissue.** Crude protein extracts were isolated from embryo tissue of muskmelon seeds imbibed for 2, 4, 8, 12, 16, 20, 24, 28, 32, or 48 h. 20  $\mu$ L crude protein from each sample were subjected to electrophoresis as in Fig. 2-3.



**Figure 2-8. Native-PAGE and activity staining for an acidic chitinase isoform from different sections of endosperm tissue.** Muskmelon seeds were imbibed for 12 h and dissected into embryonic axis (EA), cotyledon (C), the endosperm tip adjacent to the embryonic axis (NT), and the remaining endosperm tissue (N). Crude protein extracts were isolated from each section and 20  $\mu$ L were subjected to electrophoresis as described in Fig. 2-3.



**Figure 2-9. Native-PAGE and activity staining for basic chitinase isoform from muskmelon embryo tissue.** Muskmelon seeds were imbibed for 2, 4, 12, 16, 20, 24, 28, 32 and 48 h and dissected into embryo and endosperm tissue. Crude protein was extracted from embryo tissue, and 20  $\mu$ L from each sample was subjected to electrophoresis as described in Fig. 2-4.



**Figure 2-10. Native-PAGE and activity staining for basic chitinase isoforms from embryonic axis (4, 12 h) or radicle tissue (24, 32 and 48h).** Muskmelon seeds were imbibed for 4, 12, 24, 36, or 48 h and crude protein extracts were isolated. 20  $\mu$ L from each sample was subjected to electrophoresis as described in Fig. 2-4.

## **CHAPTER THREE**

### **CLONING OF CLASS II AND CLASS III CHITINASE GENES EXPRESSED IN GERMINATED MUSKMELON SEEDS**

#### **Materials and Methods**

##### **DNA Isolation**

Genomic DNA was isolated from leaves of muskmelon seedlings using the methods described by Dellaporta et al. (1983). Briefly, 1 g of fresh leaf tissue was ground in liquid nitrogen and homogenized in DNA extraction buffer (50 mM Tris, pH 8, 10 mM EDTA, 100 mM NaCl, 10 mM  $\beta$ -mercaptoethanol, 1.0% sodium dodecyl sulfate (SDS)) and centrifuged at 10,000g for 10 min. The supernatant was collected, and contaminants such as carbohydrates and proteins were precipitated by the addition of 2 mL of 5M potassium acetate after centrifugation. The resulting supernatant containing DNA and RNA was treated with RNase and then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, Sigma). The supernatant was mixed with 0.54 volume (the final volume of the supernatant) of 100% isopropanol and incubated

overnight at  $-20^{\circ}\text{C}$ . The precipitated DNA was resuspended in TE buffer and quantified spectrophotometrically.

### RNA Extraction

Total RNA was extracted from seeds or seed tissues (embryo or radicle) of imbibed seeds or seeds after radicle emergence using a standard phenol-SDS extraction method (Sambrook et al., 1989). Briefly, seeds or seed tissues were ground to a fine powder in liquid nitrogen. The tissue powder was immediately transferred to a 50 mL centrifuge tube containing 10 mL of TLE grinding buffer (0.18 M Tris, 0.09 M LiCl, 4.5 mM EDTA, 1% SDS, pH 8.2, 10  $\mu\text{L}$   $\beta$ -mercaptoethanol), 0.9 mL 2 M sodium acetate (pH 4.0) and 10 mL phenol-chloroform (5:1, Sigma). The solution was vortexed and homogenized with a tissue homogenizer on ice for a total of 3 min, shaken in an orbital shaker at 250 rpm at room temperature for 10 min, and centrifuged at 10,000g for 20 min. The supernatant was extracted with 10 mL phenol-chloroform-isoamyl-alcohol (25:24:1, Sigma). After centrifugation at 10,000g for 20 min, the supernatant was extracted with an equal volume of chloroform. The RNA was precipitated from the final supernatant by the addition of 0.5 volume (the final volume of supernatant) of 8 M LiCl and incubated overnight at  $-20^{\circ}\text{C}$ . Precipitated RNA was collected by centrifugation at 10,000g for 30 min. The resulting pellet was washed with 80% ethanol, dried, and resuspended in diethyl-pyrocabonate (DEPC)-treated water.

### Design of Degenerate Primers for Chitinases

Degenerate primers were designed based on conserved domain within known chitinases from different plant species such as rice, barley, tomato, tobacco, cotton, grape, alfalfa, bean, orange, etc. A phylogenetic tree of chitinases was created using the Clustal method (Thompson et al., 1994). Sequence alignment was performed for two branches of the tree. The first sequence alignment was performed for fifteen protein sequences of class I or II chitinases from rice, barley, tomato, tobacco, tomato, cotton, grape, alfalfa, orange, and bean with accession numbers AB018248, AB012855, X87109, 20202, L34211, 19191, 19845, A16119, A21091, 1729760, Z54234, Y10373, Z55452, Z70032, and 388509, respectively. Two conserved regions (A A F L/F A/G Q T and W F/L W M T A/P Q/R) were found from the sequence alignment for degenerate primer design. The distance between these conserved regions was about 113 amino acid residues. The degenerate primers for these two regions were an upstream primer (5'-GCC GCY TTY YTB GCK CAR AC-3') and a downstream primer (5'-TGN GSN GTC ATC CAY AAC CA-3'), respectively (R = A+G, Y= C+T, N = A+C+T+G, S = C+G, B = T+C+G, K = T+ G).

The second sequence alignment was performed for eight protein sequences of class III chitinases all from rice with accession numbers AJ007701, AJ010397, D55713, D49953, 17942, S31763, 99621 and D55711. Two well-conserved regions (A I/V Y W G Q N and W V Q F Y N N) were found during the sequence alignment. These regions

were used to design degenerate primers. The distance between these conserved regions was about 170 amino acid residues. The degenerate primers were an upstream primer (5'-GGC RTB TAY TGG GGN CAR AA-3') and a downstream primer (5'-GGR TTR TTR TAR AAY TGN ACC ACA-3'). These degenerate primers were synthesized by Life Technologies and were used for RT-PCR reactions.

### RT-PCR

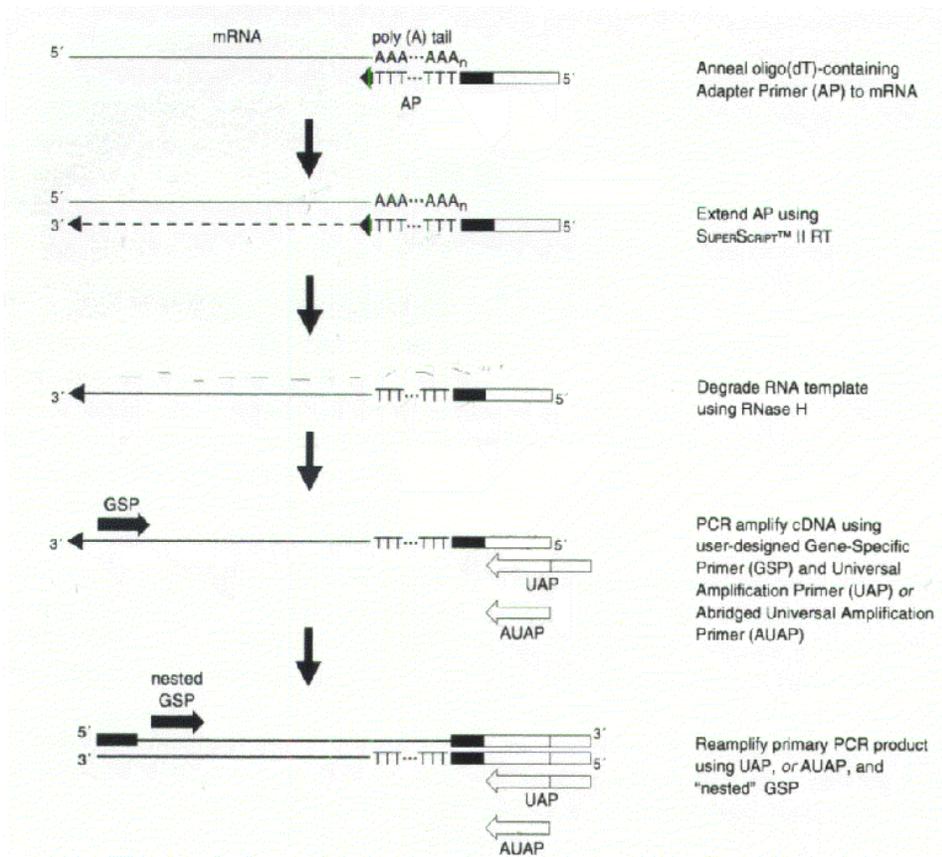
The first step in the isolation of muskmelon chitinase cDNAs was to amplify a portion of chitinase cDNAs through PCR using degenerate oligonucleotide primers. Total RNA was isolated from young developing seeds at 10, 20, and 30 DAA, or mature seeds imbibed for 16, 24, 32 or 48 h. The RNA from each stage was used for reverse transcription. The cDNA from each RNA sample was synthesized according to protocol provided by the manufacturer (RETROscript<sup>TM</sup>, Ambion). Total RNA (2 µg), Oligo (dT) (50 µM, 2 µL), dNTP mix (2.5 mM, 4 µL), and DEPC-treated water (8 µL) were mixed and heated at 70°C for 3 min and chilled on ice for 2 min. Then 10XRT-PCR buffer (2 µL), RNase inhibitor (1 µL), and reverse transcriptase from *Murine leukemia* virus were added sequentially and mixed well. The resulting mixture was incubated at 42°C for 1 h, and the reaction was terminated by heating at 95°C for 5 min and then stored at -20°C before use as templates for PCR amplification

Unless otherwise indicated, all PCR was performed according to the following program: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and then extension at 72°C for 1 min (32 cycles) in a Robocycle Gradient Thermocycler (Stratagene). The PCR products with expected sizes were cloned into pGME ®-T Easy Vector (Promega). *E. coli* cells (JM109, Promega ) were used for transformation according to the supplier's instructions. Blue/white screening was used to identify positive clones growing on the LB plate with ampicillin in the presence of 0.5 mM IPTG and 80 µg/mL X-Gal. Restriction digestion of plasmid DNA isolated from transferred cells confirmed insertion of the cloned fragments (Sambrook et al., 1989). The cloned cDNAs were sequenced by the sequencing facility at Virginia Tech. The accuracy of sequence was confirmed by sequencing the provided templates from each end using both SP6 and T7 sequencing primers. The resulting sequences were compared for homology by BLAST with chitinases in the database of the National Center of Biotechnology Information (NCBI).

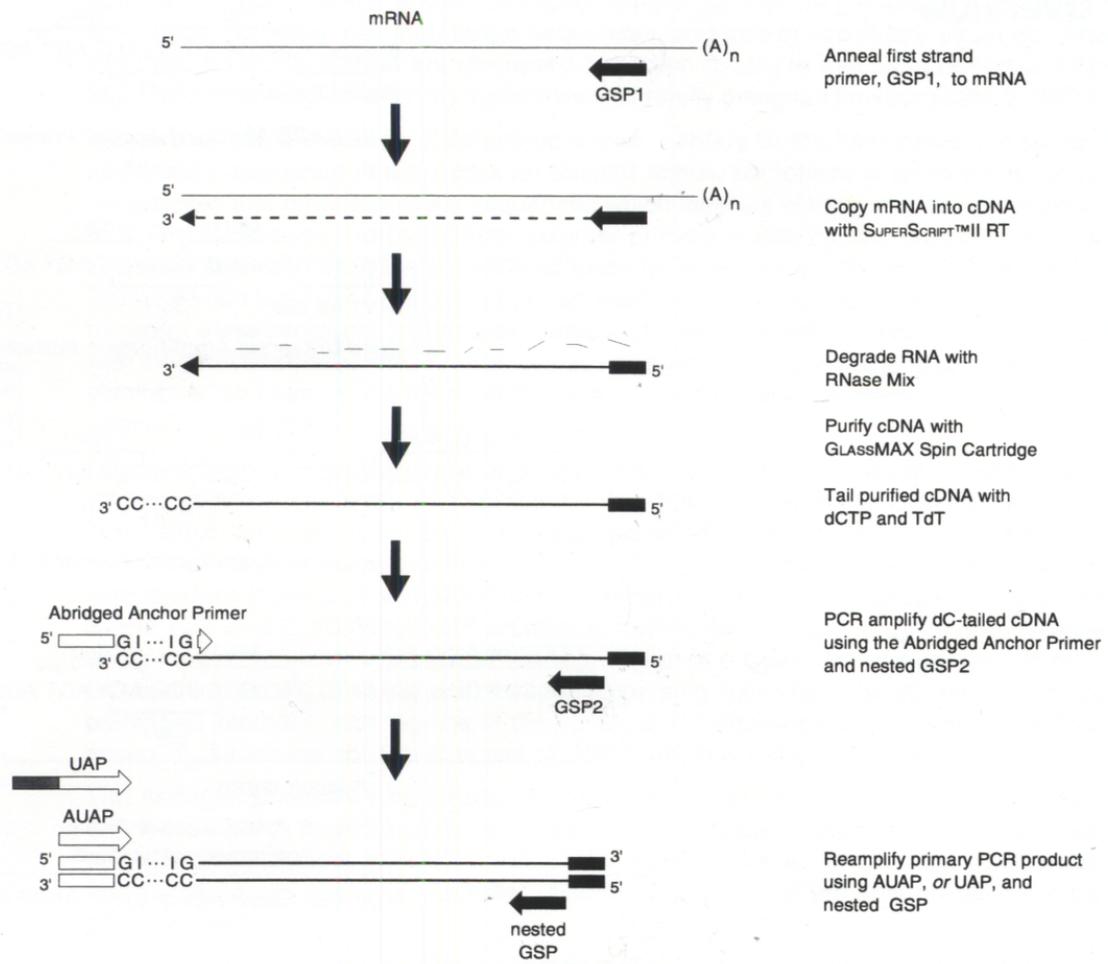
### 3'RACE and 5' RACE

The 3' and 5' ends of the RT-PCR products obtained above were amplified by the 3' RACE (Rapid Amplification of cDNA Ends) (Diagram 3-1) and the 5' RACE procedure (Diagram 3-2). Primers designed for both procedures are listed in Table 3-1 and Table 3-2. For the convenience of description, the cloned 540 bp fragment and 310 bp fragment from RT-PCR products will be referred to as putative *Cmchi1* and *Cmchi2*, respectively. Gene-specific primers for amplification of cDNA ends were synthesized

based on the nucleotide sequence of the RT-PCR products. The resulting PCR products were cloned and sequenced.



**Diagram 3-1. Overview of 3' RACE procedure (Life Technology)**



**Diagram 3-2. Overview of 5' RACE procedure (Life Technology)**

**Table 3-1. Primers and templates for amplifying 3' and 5' end of *Cmchi1* cDNA**

	Primers for RT or PCR	Template
3' RACE		
RT	Oligo(dT) <sub>30</sub>	RNA isolated from seeds imbibed for 48h
Primary PCR	RACE Adapter Primer (5'-GGC CAC GCG TCG ACT AGT AC [T] <sub>17</sub> -3') Gene specific primer 48AP 5'-AAG GCT CTC TTG CAT CCA CC-3'	cDNA synthesized from RT
Nested PCR	Universal Amplification Primer (UAP) (5'-CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC-3') Gene specific primer 48EM 5'-GTC AAA GTC CTC CTC TCT ATC G-3')	Primary PCR products
5' RACE		
RT	Gene specific primer 48GSP1 5'-TTC AAA ACA GCA GCA CCG A -3'	RNA isolated from seeds imbibed for 48h
Primary PCR	Abridged Anchor Primer (AAP) (5'-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3') Gene specific primer 48GSP2 5'-CGA TAG AGA GGA GGA CTT TG-3'	cDNA synthesized from RT
Nested PCR	Abridged Universal Amplification Primer (AUAP) 5'-GGC CAC GCG TCG ACT AGT AC-3' Gene specific primer 48GSP3 5'-GAC GAA CTC GTA GTT TCC AG-3'	Primary PCR products

**Table 3-2. Primers and templates for amplifying 3' and 5' end of *Cmchi2* cDNA**

	Primers for RT or PCR	Template
3' RACE		
RT	Oligo(dT) <sub>30</sub>	RNA isolated from seeds imbibed for 24h
Primary PCR	RACE Adapter Primer (5'-GGC CAC GCG TCG ACT AGT AC [T] <sub>17</sub> -3') Gene specific primer 24AP 5'-GCT AAC TGC ACC AGA TGG TC-3'	cDNA synthesized from RT
Nested PCR	Universal Amplification Primer (UAP) (5'-CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC-3') Gene-specific primer 24DM 5'-ATG GTC GTG GAC CAA TGC AA-3'	Primary PCR products
5' RACE		
RT	Gene-specific primer 24 GSP1 5'-CTA AAT CTG GGT TCT TCA GC-3'	RNA isolated from seeds imbibed for 24h
Primary PCR	Abridged Anchor Primer (AAP) (5'-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3') Gene-specific primer 24GSP2 5'-TTG CAT TGG TCC ACG ACC AT-3'	cDNA synthesized from RT
Nested PCR	Abridged Universal Amplification Primer (AUAP) 5'-GGC CAC GCG TCG ACT AGT AC-3' Gene-specific primer 24 GSP3 5'-TGG TGC AGT TAG CCA TCC TC-3'	Primary PCR products

## Cloning of the Full Lengths of Both *Cmchi1* and *Cmchi2* cDNAs and the Partial Genomic

### *Cmchi2* Clone

Sequences generated from both 3' and 5' RACE procedures for both putative *Cmchi1* and *Cmchi2* were used to design the primers to obtain the full length of cDNAs for both genes. The full length cDNA for *Cmchi1* was obtained by PCR using the cDNA synthesized from RNA isolated from seeds imbibed for 48 h as the template, an upstream primer 48FW (close to 5' end) (5'-CAC AAA CTC CAA CCA AAG CTC-3') and a downstream primer 48RV (close to 3' end) (5'-AGC TTA ATT CAG CCG ATG CTG – 3'). PCR conditions were the same as above except for the annealing temperature (58°C). The resulting 1 kb fragment was then cloned and sequenced as described above.

The full length cDNA for *Cmchi2* cDNA was amplified by PCR using an upstream primer 24FW1 (close the 5' end) (5'-CGA ATT TAA AAC TCA GAG AGA AAA-3'), a downstream primer 24RV (close to 3' end) (5'-TCC CTC CTA TCT CAT CTC GT-3') and the cDNA synthesized from RNA isolated from seeds imbibed for 24 h as the template. Both 24FW1 and PCR conditions were the same as previously described. The resulting 1 kb fragment was then cloned and sequenced as described above. The partial genomic putative *Cmchi2* fragment was also amplified via PCR using genomic DNA (100 ng) as the template and the same primers as mentioned above. The PCR conditions were the same as above except for the annealing temperature (50°C). The resulting 1.3 kb fragment was cloned and sequenced.

## DNA Probe Synthesis

Digoxigenin (DIG)-labeled DNA probes were synthesized according to the manufacturer's protocol (PCR DIG Probe Synthesis Kit, Boehringer Mannheim). Templates for the synthesis of the probes were prepared by PCR using the vector containing the full length cDNA for *Cmchi1* and *Cmchi2* as templates. The resulting PCR products were then purified from 1.0% agarose gel and used as templates for synthesis of the probes with a PCR DIG Probe synthesis kit. The PCR reactions (50  $\mu$ L) contained 10X PCR buffer (5  $\mu$ L), enzyme mix (2.6 U), PCR DIG probe synthesis mix (200  $\mu$ M dATP, 200  $\mu$ M dCTP, 200  $\mu$ M dGTP, 200  $\mu$ M dTTP and 7  $\mu$ M DIG-11-dUTP), template (100 ng) and primers (200 nM each). The probe for *Cmch1* cDNA was synthesized using an upstream primer 48FW (5'-CAC AAA CTC CAA CCA AAG CTC-3') and a downstream primer 48 RV (5'-AGC TTA ATT CAG CCG ATG CTG -3'). The primers used for synthesis of the probe for *Cmchi2* cDNA were an upstream primer 24FW1 (5'-CGA ATT TAA AAC TCA GAG AGA AAA-3') and a downstream primer 24RV (5'-TCC CTC CTA TCT CAT CTC GT-3'). PCR conditions (32 cycles) were 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. Control PCR reactions were run by including all the components used for the synthesis of the probe except DIG-UTP. The success of the probe synthesis was assessed via agarose gel electrophoresis comparing the probe side by side with the control PCR product. The position of DIG-labeled probe on

the gel is shifted when compared to that of the unlabeled control cDNA as migration of the DIG-labeled probe is retarded.

### Southern Hybridization

Genomic DNA (20 µg) was digested at 37°C for 3 h with restriction enzymes BamH I, EcoR I and Hind III (Promega). The digested DNA and DIG-labeled DNA Marker (Boehringer Mannheim) were subjected to electrophoresis through agarose gel (1% Seakem Gold, FMC) for 6 h at 70V. The gel was then denatured in 1.5 M NaCl, 0.5 M NaOH for 30 min with gentle agitation followed by soaking in neutralizing buffer 0.5 M Tris-HCl, pH 7.0, 1.5 M NaCl for 30 min. The gel was then soaked in transfer buffer (20X SSC) for 30 min. The gel was next transferred to a nylon membrane (Schleicher & Schuell) using the rapid downward transfer system (TurboBlotter, Schleicher & Schuell) for 8 h. The DNA blotted membrane was then cross-linked using the optimal UV scale (UV Crosslinker, Fisher).

The membrane was prehybridized with a commercial hybridization buffer Ultrahyb (Ambion) at 42°C for 1 h and then hybridized at 42°C overnight with DIG-labeled probe. The membrane was washed twice for 15 min in a low stringency washing solution (2X SSC, 0.1% SDS) at room temperature and twice in a high stringency washing solution (0.1X SSC, 0.1% SDS) at 42°C.

### Detection of Hybridization Signals

A chemiluminescent detection system was used to detect the hybridization signal. Briefly, the membrane was washed in washing buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.3% (v/v) Tween ® 20) for 2 min and blocked for 1 h in blocking solution (Boehringer Mannheim). After blocking, the membrane was incubated with alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim, 1:10,000 dilution) for 30 min. After washing the membrane twice (15 min each) with washing buffer, the membrane was soaked briefly in the detection buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). The membrane was then incubated with the chemiluminescent substrate CDP-Star™ for 5 min. The signal was detected on X-ray film (Biomax MR, Kodak) after 2 to 20 min exposures.

## **Results**

### RT-PCR

Using degenerate primers designed for class III chitinases, a distinct fragment with the expected size of 540 bp was amplified from cDNA synthesized from RNA isolated from muskmelon seeds imbibed for 48 h (Fig. 3-1A). Using degenerate primers designed for class I or II chitinases, a distinct fragment with an expected size of 310 bp was also amplified from cDNA synthesized from RNA isolated from muskmelon seeds

imbibed for 24 h (Fig. 3-1B). These PCR products were cloned and sequenced. The resulting sequences were compared with other known chitinase sequences in the database. The nucleotide sequence of 540-bp fragment shows 92% identity to class III chitinases from cucumber, while the nucleotide sequence of 310-bp fragment shows 72 % identity to class I chitinase from pumpkin. Other sequences identified from the database are also chitinases. This indicates that these RT-PCR fragments correspond to partial chitinase genes from muskmelon seeds after radicle emergence. The 540-bp fragment was designated as *Cmchi1*, while the 310-bp fragment was designated as *Cmchi2*.

### 3' RACE

The 3' end of both *Cmchi1* and *Cmchi2* were amplified by the RACE procedure. Using cDNA synthesized from RNA isolated from muskmelon seeds imbibed for 48 h as template, 3'RACE adapter primer provided by the Kit, and the gene-specific primer 48AP which is designed based upon the sequence of *Cmchi1* (RT-PCR product), primary PCR was performed. Multiple products were amplified from the primary PCR (Lane P1, Fig. 3-2A). In the case of obtaining the 3' end of *Cmchi2*, cDNA was synthesized from RNA isolated from seeds imbibed for 24 h and used as the template for PCR in combination with a gene specific primer 24 AP and the 3' RACE adapter primer. Multiple products were also produced from primary PCR (Lane P2, Fig. 3-2A).

To enrich the fragment of interest, nested PCR was performed. A gene specific primer 48EM was designed downstream of the first gene-specific primer 48AP for

*Cmchi1*, while a gene specific primer 24DM was designed downstream of the first gene-specific primer 24AP for *Cmchi2*. Nested PCR was performed to amplify the 3' end of *Cmchi1* using the primer pair 48EM and 3' RACE adapter primer and the primary PCR reaction as the template. Nested PCR was also performed to amplify the 3' end of *Cmchi2* using the primer pair 24DM and 3' RACE adapter primer and the primary PCR reaction as template. As revealed by agarose gel electrophoresis, a high level of a single product was obtained from each nested PCR reaction (Fig. 3-2B). The nested PCR products were cloned and sequenced. The resulting sequences contained poly (A) at the 3' ends of both *Cmchi1* and *Cmchi2*. Database searches confirmed that the cloned fragments contained both the sequence identical to *Cmchi1* or *Cmchi2* and the 3' end sequence obtained by the 3' RACE procedure.

#### 5'RACE

To obtain 5' ends of both *Cmchi1* and *Cmchi2*, 5'RACE procedure was performed. In the case of amplifying the 5' end of *Cmchi1*, the primary PCR produced one fragment using the second gene-specific primer 48GSP2 and the Abridged Anchor primer (AAP) (Fig. 3-3). Nested PCR produced with the gene-specific primer 48GSP3 and the Abridged Universal Amplification Primer (AUAP) produced a smaller fragment. This fragment was cloned and sequenced.

The primary PCR reaction for *Cmchi2* produced more than two fragments, with two major fragments showing on the gel when using the second gene specific primer

24GSP2 and AAP (Lane P, Fig. 3-4). Nested PCR produced two smaller fragments that were very close to each other when using the third gene specific primer 24GSP3 and AUAP (Lane N, Fig. 3-4). The nested PCR product was also cloned and sequenced. Database searches confirmed that the cloned fragments contained both the sequence identical to the middle part of *Cmchi1* or *Cmchi2* and the 5' end sequence obtained by the 5' RACE procedure.

#### Cloning the full-lengths of both *Cmchi1* and *Cmchi2*

Sequences generated from both 3' and 5'RACE procedures for *Cmchi1* and *Cmchi2* were used to design the primers to obtain the full length of both *Cmchi1* and *Cmchi2* cDNA. The full length of *Cmchi1* cDNA with a size of about 1 kb was amplified by PCR using an upstream primer 48 FW and a downstream primer 48 RV (Fig. 3-5). Using genomic DNA as template, PCR also produced a fragment the same size as the fragment amplified using cDNA as a template (Lane G, Fig. 3-6). Figure 3-8 also showed no product when using water as template for PCR, indicating contamination was ruled out from the primer and other reagents (lane X, Fig. 3-6).

The full length of *Cmchi2* cDNA with a size of about 1 kb was also amplified by PCR using an upstream primer (24FW1) and a downstream primer (24RV) (Fig. 3-7). The fragment amplified from the *Cmchi2* cDNA template was smaller than the fragment amplified from genomic DNA template (Fig. 3-9). This indicates that *Cmchi2* has

introns. Later sequence information resulting from the partial genomic clone revealed that the *Cmchi2* genomic clone has two introns in its coding region.

#### Characteristics of *Cmchi1* cDNA

The complete *Cmchi1* cDNA sequence and deduced amino acid sequence is shown in Fig. 3-8. It is 991 bp in length and exhibits a 5' untranslated region of 39 bp an ATG initiation codon at position 40, followed by an open reading frame of 873 bp. The stop codon TGA starts at position 917 and is followed by a 3' untranslated region of 73 bp.

The Protean program (Lasergene) was used to calculate biochemical properties of the derived amino acid sequence of the coding region. The protein was predicted to have a molecular mass of 30,784 Da. The isoelectric point was predicted to be 4.19 with a charge of -11.76 at neutral pH. The protein sequence was analyzed using the program SignalP (Nielsen et al., 1997) to determine if a signal peptide was present. A cleavage site was predicted between amino acid residue 25 and 26 (Ala-Ala). The derived protein sequence also contains a functional domain of 266 amino acid residues.

#### Characteristics of *Cmchi2* cDNA and Partial Genomic DNA Clone

The complete *Cmchi2* cDNA sequence and deduced amino acid sequence is shown in Fig. 3-9. The cDNA is 1038 bp in length and consists of a 5' untranslated region of 24 bp, an ATG initiation codon at position 25, followed by an open reading frame of 834 bp. The stop codon TAA starts at position 841 and is followed by a 3'

untranslated region of 180 bp. The deduced protein was predicted to have a molecular mass of 30,099 Da. The isoelectric point was predicted to be 9.28 with a charge of 13.32 at neutral pH. The program SignalP also predicted the existence of a signal peptide. The cleavage site was predicted between amino acid 20 and 21 (Gly-Val). The derived amino acid sequence of the coding region contains a functional domain of 252 amino acid residues.

The partial genomic DNA sequence for *Cmchi2* is shown Fig. 3-10. It is 1394 bp in length. It contains three exons and two introns. The first exon starts at nucleotide 118 and ends at nucleotide 412. The second exon starts at nucleotide 606 and ends at nucleotide 755. The third exon starts at nucleotide 846 and ends at nucleotide 1219. The first intron starts at nucleotide 413 and ends at nucleotide 605, and the second intron starts at nucleotide 756 and ends at nucleotide at 845.

#### Homology Search for *Cmchi1* and *Cmchi2* cDNA Sequences and Protein Sequences

The *Cmchi1* and *Cmchi2* nucleotide sequences and predicted amino acid sequences were compared to the non-redundant database using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). In a search against the non-redundant GeneBank nucleotide databases, the nucleotide sequences of *Cmchi1* and *Cmchi2* show homology to chitinases from many plant species. *Cmchi1* shows 92% identity to a class III chitinase gene from *Cucumis sativus*, while *Cmchi2* shows 72% identity to a class I chitinase gene from pumpkin.

A comparison of the *Cmchi1* protein sequence against the Swiss Prot protein database showed 21 proteins with significant alignment to the *Cmchi1* protein sequence. The 10 highest scoring sequences (Table 3-3) were plant class III chitinases. The next eight highest scoring sequences were from fungi. The 18 highest scoring sequences were imported into DNA star format and aligned with the *Cmchi1* protein sequence using the Clustal method (Thompson et al., 1994) in MegAlign (Lasergene). From this alignment, a phylogenetic tree was generated (Fig. 3-11). *Cmchi1* protein grouped most closely with Accession number P17541, a class III chitinase protein from cucumber. The amino acid sequence of *Cmchi1* protein was then compared with other class III chitinases from *Cucumis sativus* (Mettraus et al., 1989), *Vigna angularis* (Ishige et al., 1991), and *Hevea brasillensis* (Jekel et al., 1990) (Fig. 3-12). The level of identity of amino acids varied from 94.9% to 51.9%. This indicates that *Cmchi1* encodes a class III chitinase from germinated muskmelon seeds.

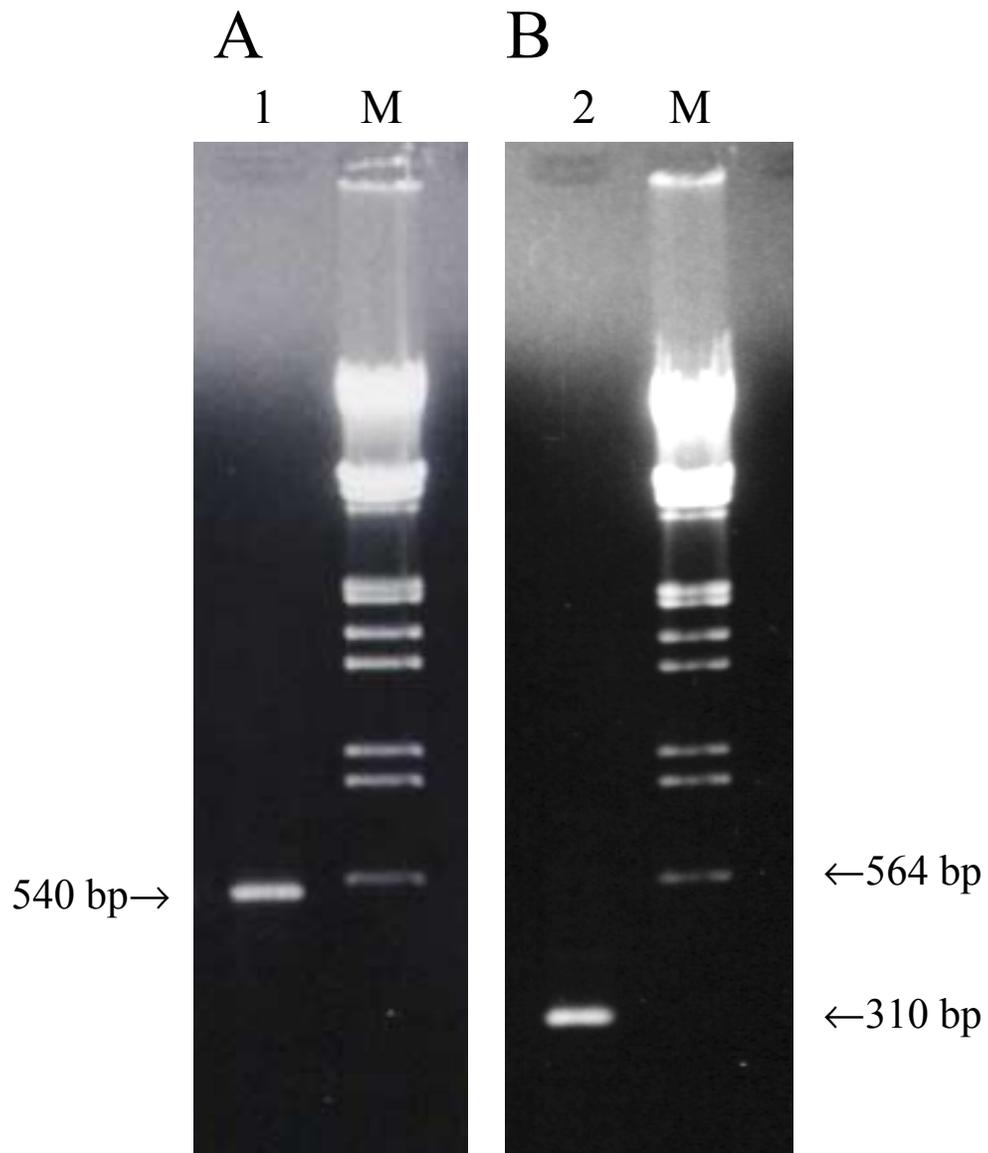
A comparison of the *Cmchi2* protein sequence against Swiss Prot protein database showed 54 proteins showing identity to the *Cmchi2* protein sequence. The 19 highest scoring sequences (Table 3-4) were plant class I or II chitinases. The 19 highest scoring sequences were also imported into DNA star format and aligned with the *Cmchi2* protein sequence using the Clustal method in MegAlign (Lasergene). A phylogenetic tree was generated from this alignment (Fig. 3-13). *Cmchi2* protein does not group closely with other chitinases, as it lacks the chitin-binding domain. This indicates that *Cmchi2* might encode a class II chitinase, since class II chitinases do not contain a chitin-binding

domain. The amino acid sequence of *Cmchi2* protein was then compared with other known II chitinases from *Hordeum vulgare* (Leah et al., 1991), *Lycopersicon esculentum* (Danhash et al., 1993) and *Nicotiana tobacum* (Payne et al., 1990), *Petunia x hybrida* (Linthorst et al., 1990) and *Solanum tuberosum* (Wemmer et al., 1994) (Fig. 3-14). The level of identity varied from 51.9 to 45.5%.

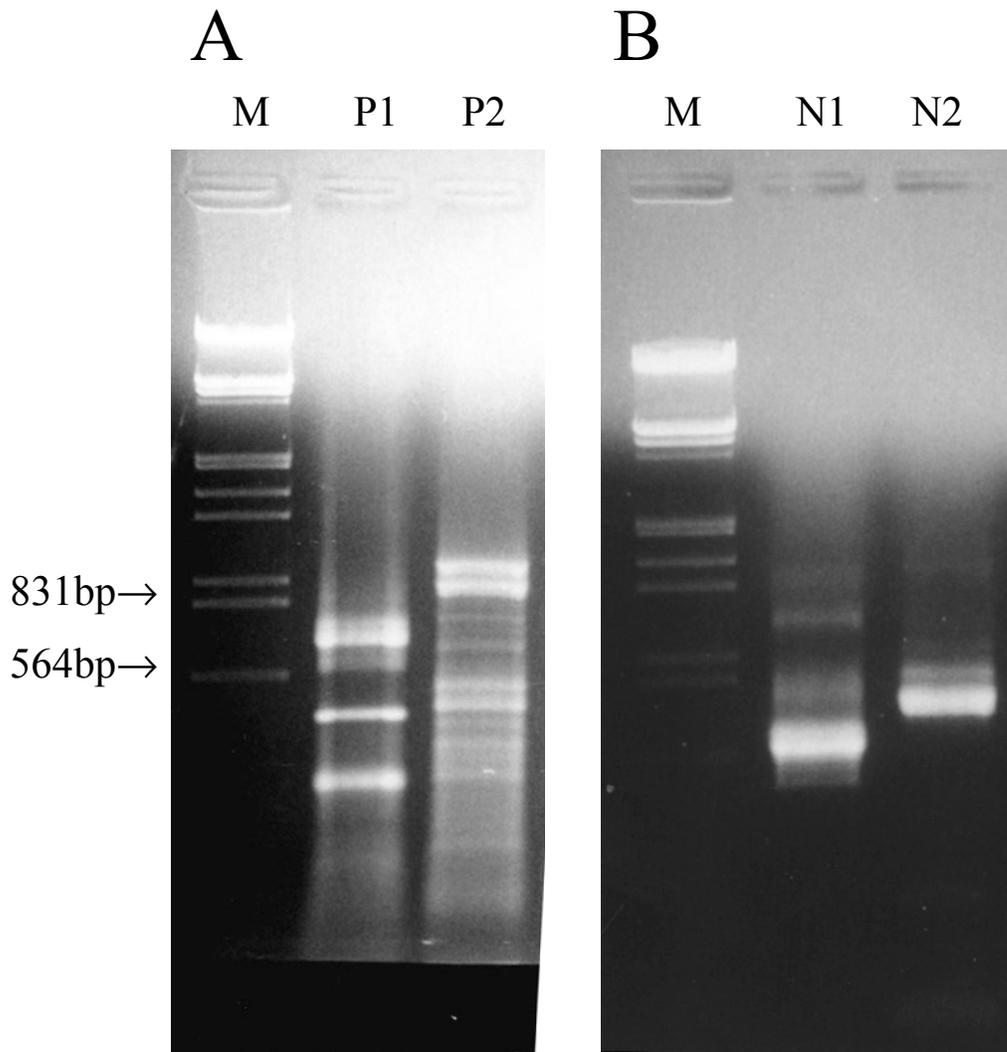
#### Southern Blot Analysis

In order to assess the presence of other genes identical or similar to *Cmchi1* or *Cmchi2* in the muskmelon genome, Southern analysis was performed. Two hybridization signals were detected when genomic DNA was digested by restriction enzymes BamH I, EcoR I and Hind III and probed with DIG-labeled *Cmchi1* cDNA (Fig. 3-15). One hybridization signal was produced when genomic DNA was digested by restriction enzyme BamH I, EcoR I, and Hind III and probed with DIG-labeled *Cmchi2* cDNA (Fig. 3-16).

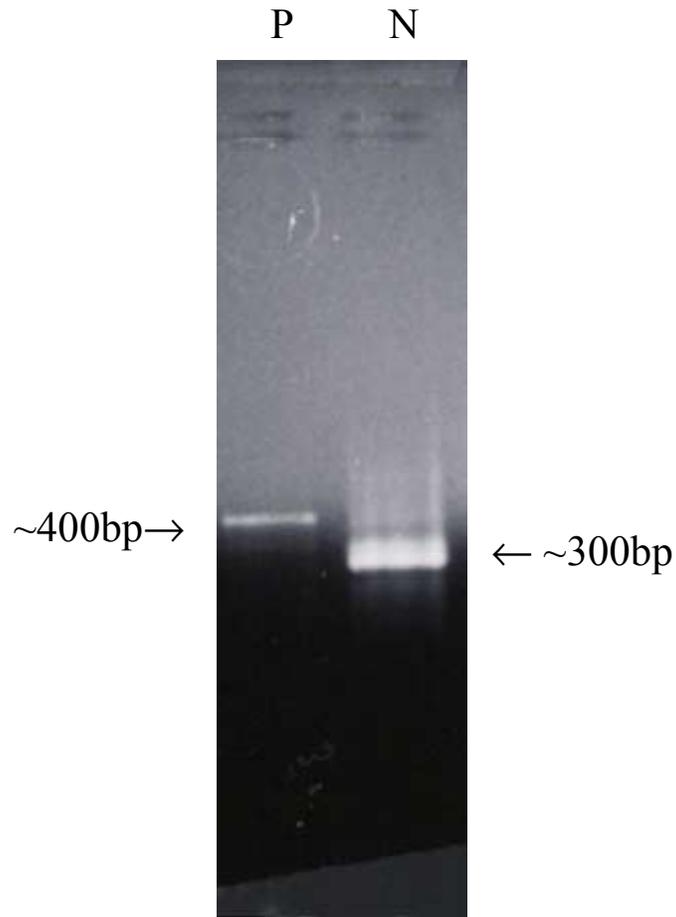
## **Figures**



**Figure 3-1. RT-PCR products amplified from RNA isolated from germinated muskmelon seeds.** In (A), the 540 bp fragment in lane 1 was amplified from cDNA synthesized from RNA isolated from seeds imbibed for 48 h using degenerate primers designed for class III chitinases. In (B), the 310 bp fragment in lane 2 was amplified from cDNA synthesized from RNA isolated from seeds imbibed for 24 h using degenerate primers designed for class I or II chitinases. M = DNA-size marker.

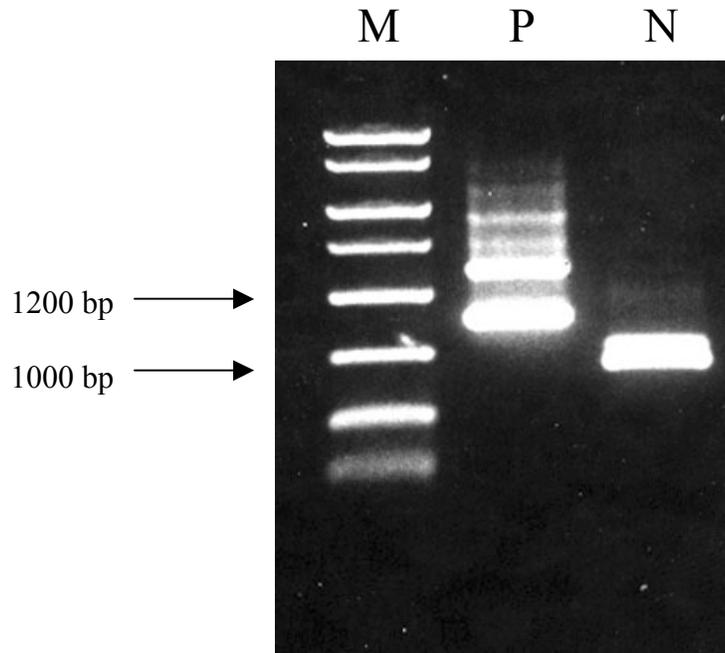


**Figure 3-2. PCR products for both putative *Cmchi1* and *Cmchi2* cDNA generated by the 3'RACE procedure.** In (A), shown in lanes P1 and P2 are the primary PCR products using *Cmchi1* gene-specific primer 24AP (P1) or *Cmchi2* gene-specific primer 48AP (P2) and the adapter primer provided with the Kit. In (B), shown in lane N1 and N2 are nested PCR products using the gene-specific primer 24DM (N1) or 48EM (N2) and the Universal Amplification Primer (UAP). The templates for N1 and N2 are PCR products shown in P1 and P2, respectively. M = DNA-size marker



**Figure 3-3. PCR products for *Cmchi1* generated by the 5' RACE procedure.**

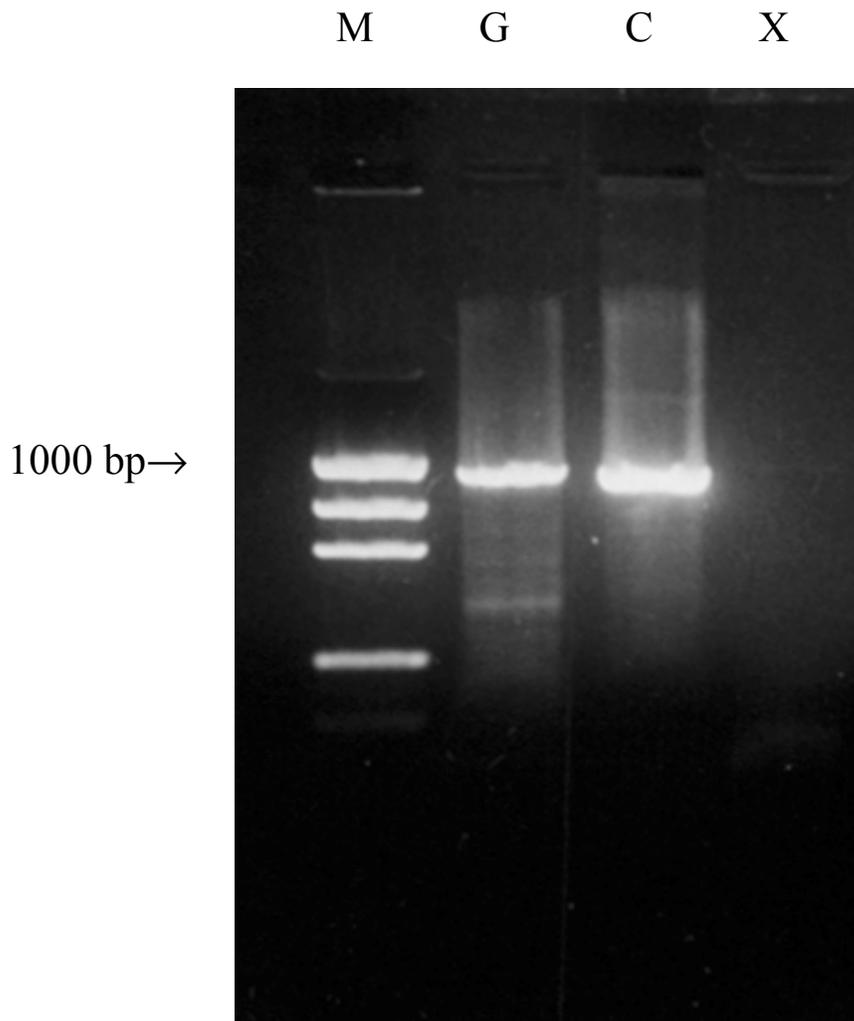
Shown in lane P is the PCR product amplified using template RT product (synthesized from RNA isolated from seeds imbibed for 48 h, the first gene-specific primer 48GSP1) and the primer pair (the second gene-specific primer 48GSP2 and the Abridged Anchor Primer (AAP) provided with the kit). Shown in lane N is the nested PCR product using the template shown in lane P, the third gene specific primer 48GSP3, and the Abridged Universal Amplification primer (AUAP) provided with the Kit.



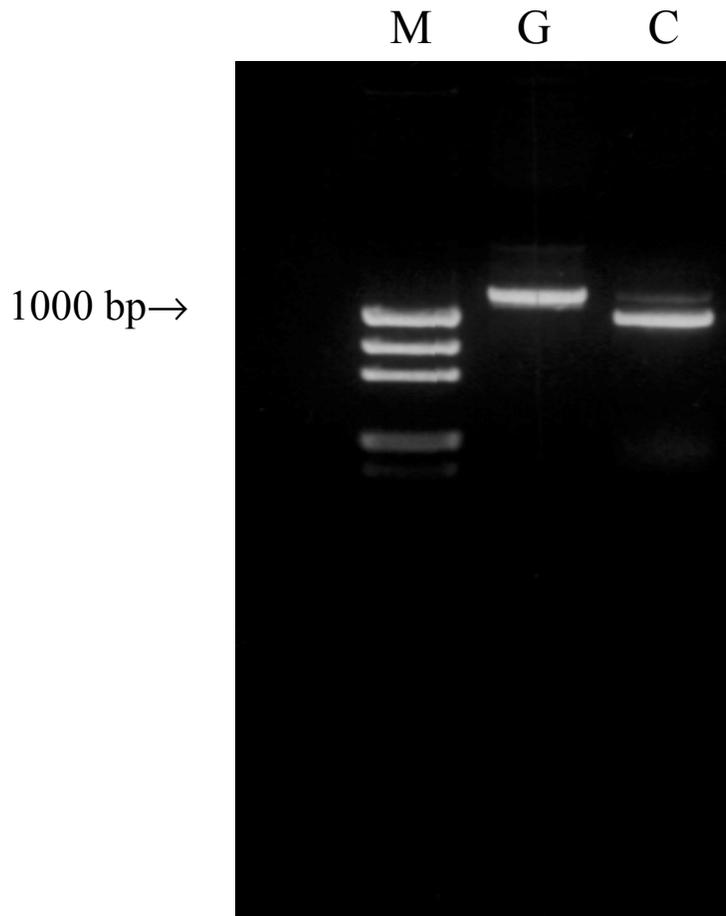
**Figure 3-4. PCR products for *Cmchi2* generated by the 5' RACE procedure.** Shown in lane P is the PCR product amplified using template RT product (synthesized from RNA isolated from seeds imbibed for 24 h and the first gene-specific primer 24GSP1), the primer pair (the second gene-specific primer 24GSP2 and the Abridged Anchor Primer (AAP) provided with the kit). Shown in lane N is the nested PCR product using the template shown in lane P, the third gene-specific primer 24GSP3 and the Abridged Universal Amplification primer (AUAP) provided with the kit. M = DNA-size marker



**Figure 3-5. The full length of *Cmchi1* cDNA.** Shown in lane C is the PCR product amplified using the gene-specific primer pair 48FW and 48RV and the template cDNA synthesized from RNA isolated from seeds imbibed for 48 h. M = DNA-size marker.



**Figure 3-6. The full length of *Cmchi1* amplified from cDNA or genomic DNA template.** Shown in lane C is the PCR product amplified from cDNA synthesized from RNA isolated from seeds imbibed for 48 h and the gen-specific primer pair-48FW and 48RV. Shown in lane G is the PCR product amplified from template genomic DNA isolated from leaves of young muskmelon seedlings and the gene-specific primer pair-48FW and 48RV. Shown in X is the PCR reaction using water as template and the gene-specific primer pair 48FW and 48RV. M = DNA-size marker



**Figure 3-7. The full length of *Cmchi2* cDNA amplified from the cDNA template and the partial genomic *Cmchi2* PCR product.** Shown in lane C is the PCR product amplified from cDNA synthesized from RNA isolated from seeds imbibed for 24 h and the gene-specific primer pair-24FW1 and 24RV. Shown in lane G is the PCR product amplified from template genomic DNA isolated from leaves of young muskmelon seedlings and the gene-specific primer pair-24FW and 24RV. M = DNA-size marker.

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AAACACACAAACTCCAACCAAAGCTCTTTAAGAAATATAATGGCTGCCACAAAATAACTACGACCCTTTC 71
                                     M A A H K I T T T L S
CATCTTCTTCTCCTCTCCTCTATTTTTCCGGTCTTCCGAGGCAGCTGGAATCGGCGTGTACTGGGGTCAAA 142
    I F F L L S S I F R S S E A A G I G V Y W G Q
ACGGAAACGAAGGCTCTCTTGCATCCACCTGCGCCACTGGAAATACGAGTTCGTCAACATAGCATTTC 213
    N G N E G S L A S T C A T G N Y E F V N I A F L
TCATCCTTCGGCAGCGGTCAAACCTCCGGTCTCAACCTTGCCGGTCATTGCAACCCTGACAACAACGGTTG 284
    S S F G S G Q T P V L N L A G H C N P D N N G C
CGCCTTTTTGAGCGACGAAATAAATCTTGCCAAAGTCAAATGTCAAAGTCTCTCTATCGGGCGG 355
    A F L S D E I N S C Q S Q N V K V L L S I G G
GTGCGGGGAGTTATFCACTCTCCTCCGCCGACGATGCGAGACAAGTCGCGAACTTCCTTTGGAACAGCTAC 426
    G A G S Y S L S S A D D A R Q V A N F L W N S Y
CTCGCGGGCAGTCAGATTCCAGGCCACTCGGTGCTGCTGTTTTGAATGGTATTGATTCGATATCGAGTC 497
    L G G Q S D S R P L G A A V L N G I D F D I E S
TGGCTCGGGCCAGTTCTGGGATGTACTAGCTCAGGAGCTAAAGAGTTTTGGACAAGTCATTTTATCTGCCG 568
    G S G Q F W D V L A Q E L K S F G Q V I L S A
CGCCGAGTGTCCGATCCCAGACGCTCACCTAGACGCCGGGTCAAACCTGGACTGTTTCGATTCCGTTTGG 639
    A P Q C P I P D A H L D A A V K T G L F D S V W
GTTCAATTCTACAACAACCCGCCATGCATGTTTGAGATAACCGGACAATCTCCTGAGTTCATGGAATCA 710
    V Q F Y N N P P C M F A D N A D N L L S S W N Q
GTGGACGGCGTTTCCGATATCGAAGCTTTACATGGGATTGCCAGCGGCACCGGAGGCAGCGCCGAGCGGG 781
    W T A F P I S K L Y M G L P A A P E A A P S G
GATTATTCCGGCGGATGTGCTTATTTCTCAAGTCTTCCAACCATTAACAACTCTTCCAACCTATGGAGGA 852
    G F I P A D V L I S Q V L P T I K T S S N Y G G
GTGATGTTATGGAGTAAGCGTTTGACAATGGCTACAGCGATGCCATTAAGGCAGCATCGGCTGAATTAA 923
    V M L W S K A F B N G Y S D A I K G S I G .*
GCTTCTAAGCTTAAATTTAATTAAGCTATGAATAAACTCCAAGTATTATAATAAAAAAAAAAAAAA 991

```

**Figure 3-8. Complete cDNA sequence of *Cmchi1* and the deduced amino acid sequence.**

**The putative signal sequence is indicated by ▲ and polyadenylation site and stop codon are indicated by and \*, respectively.**

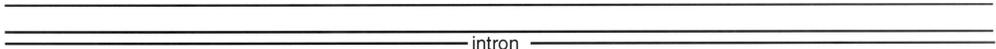
CGAATTTAAAACCTCAGAGAGAAAAATGAAGCTTTATTTCATTATTAAGTTTGGTGTGTTATGGAGATCAGAGGGTGTAGCT 90  
M K L Y S L L S L L F L G V L W R S E G ▲ V A  
TCAAGTTCAAATGACGATGTAGGAAGGCTCATTAAACGTCACAACATTCAATGCAATGTTCAAATATCAAAGGATCCACAATGTCCAAGC 180  
S S S N D D V G R L I N V T T F N A M F K Y Q K D P Q C P S  
CAAGGGTCTATAGTTACCAAGCTTTTTTAACTGCGGCTCGTTCCTTTGGTAAGTTAGGGTTCGCCACCACCGAAAACTTGCTACTCGA 270  
Q G F Y S Y Q A F L T A A R S F G K L G F A T T G K L A T R  
AAGAGGGAGTTGCTCGCTTTCCTCGCTCAAACCTCTCATCAAACACCGAGGATGGCTAACTGCACCAGATGGTCCACTTTTTTGGGGA 360  
K R E L L A F L A Q T S H Q T T G G W L T A P D G P L F W G  
TATTGCCACATAAGAGAAAAGCACTGAAGATTCTATTGCAAAGCTGATCCTAAATGGCCTTGTGCTAAAGGCCAAAAATATTATGGTCGT 450  
Y C H I R E S T E D S Y C K A D P K W P C A K G Q K Y Y G R  
GGACCAATGCAACTCAAAGGAAATCAGAACTATGGCCAAGCTGGGAAAGCGTTGGGATTGGATTGCTGAAGAACCCAGATTTAGTAGCT 540  
G P M Q L K G N Q N Y G Q A G K A L G L D L L K N P D L V A  
AAAGACCCAGTTGTATCGTTCAAGACAGCGATTGGTTTTGGATGACAGCACAGGGAATCAAACCATCGTGTATGATGTTATGGTGGGG 630  
K D P V V S F K T A I W F W M T A Q G I K P S C H D V M V G  
AAATGGAAACCCACGGAAGCTGACAAAGCTGCTAAGAGAGTCCCTGGTTACGGTGTGAGCAATATCATTGGTGAAGTGAATGTGGG 720  
K W K P T E A D K A A K R V P G Y G V V S N I I G G S E C G  
AGTGGTGTCTAATACTGATGTTGCTGATAGATTTGGATTTTACGTAAGGTAAGTACTGCAAGATGTTGGGAGTCAATCCAGGAAAACACTTGGAT 810  
S G A N T D V A D R F G F Y V R Y C K M L G V N P G K H L D  
TGCTTTTTTCAACAACCTTTCCTCGAATGTAATGCCCAATAACAACCTTCTTTAATTGTTTCAAATTTAGAAAAAGAGGCTTGAATA 900  
C F F Q Q P F T R M .\*  
AACAAATGGGAATAAAGAATCGCAGAATCGTGGCTCTCGTTGGCCTAAAAACGAGATGAGATAGGAGGAAATTGAACCACATATTTCTT 990  
AGTCTTCAACGATTATTTTATGCTGGTTGAACTAAAAAAAAAAAAAAAA 1038

**Figure 3-9. Complete cDNA sequence of *Cmchi2* and the deduced amino acid sequence. The putative signal sequence is indicated by▲ and polyadenylation site and stop codon are indicated by \_ and \*, respectively**

GTGACACTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCCCATATGGTCGACCTGCAGGCGGCCGGAATTCAC 86  
 AGTGATTCGAATTTAAAACCTCAGAGAGAAAAATGAAGCTTTATTCATTATTAAGTTTGTGTTTTGGGTGTGTTATGGAGATCAG 172  
 AGGGTGTAGCTTCAAGTTCAAATGACGATGTAGGAAGGCTCATTACGTCAACAACATCAATGCAATGTTCAAATATCAAAAGGAT 258  
 CCACAATGTCCAAGCCAAGGGTTCTATAGTTACCAAGCTTTTTAACTGCGGCTCGTTCCTTTGGTAAGTTAGGGTTCGCCACCAC 344  
 CGGAAAACCTTGCTACTCGAAAGAGGGAGTTGCTCGCTTTCTCGCTCAAACCTTCATCAAACCACCGGTTAGTAGCACTCACTTA 430



A TACTACTCTTTATATTGTTATAATAATTATTGTTAATCCAGCTAATATGATATTAATTATTGAAAATGGAGGCATCTCTTCCCAT 516



TGGTATTTAGATGTATAGCTTCATGCTATTATCTTATAAAAATCACCACAACTCTACTCATTCAATTTAAGATTTTGATACATACA 602



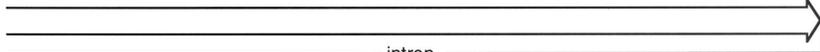
TAGGAGGATGGCTAACTGCACCAGATGGTCCACTTTTTGGGGATATTGCCACATAAGAGAAAGCACTGAAGATTCCTATTGCAAA 688



GCTGATCCTAAATGGCCTTGTGCTAAAGGCCAAAAATATTATGGTCGTGGACCAATGCAACTCAAAGGGTAAGTTAGGTTAAATAA 774



TTAATTACAGTACTCACAGATTTAATCTCTCCTCCTTAATTACAACCTTTGGTTTACAATATATTAACCGAGAAATCAGAACTATG 860

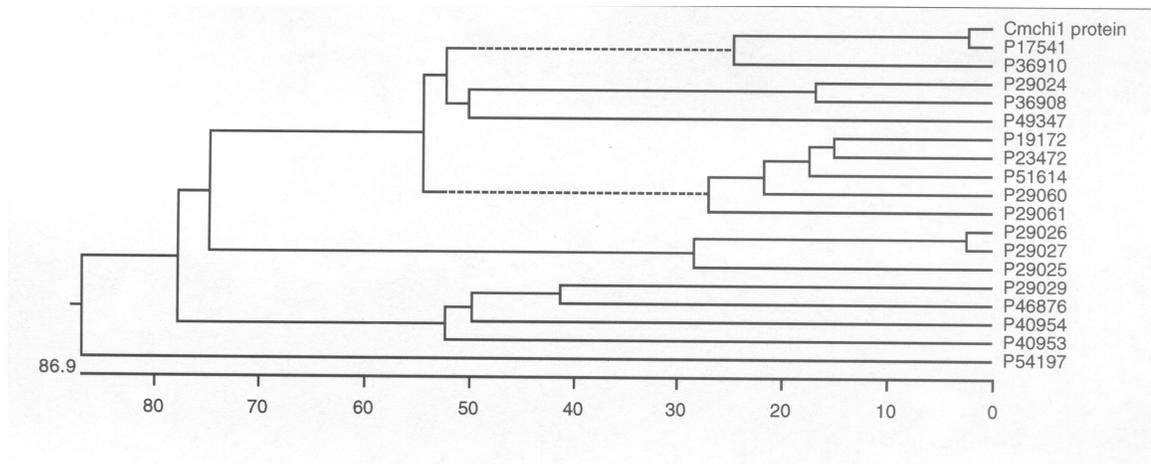


GCCAAGCTGGGAAAGCGTTGGGATTGGATTTGCTGAAGAACCCAGATTTAGTAGCTAAAGACCCAGTTGTATCGTTCAAGACAGCG 946  
 ATTTGGTTTGGATGACAGCACAGGGAATCAAACCATCGTGTCATGATGTTATGGTGGGAAATGGAAACCCACGGAAGCTGACAA 1032  
 AGCTGCTAAGAGAGTCCCTGGTTACGGTGTGTGAGCAATATCATTGGTGAAGTGAATGTGGGAGTGGTGCATAACTGATGTTG 1118  
 CTGATAGATTTGGATTTTACGTAAGGTACTGCAAGATGTTGGGAGTCAATCCAGGAAAACACTTGGATTGCTTTTTTCAACAACCT 1204  
 TTCACTCGAATGTAATGCCCAATAACAACCTTCTTAATTGTTTCAAAATTTAGAAAAGAGGCTTGTAAATAACAATGGGAATAA 1290  
 AAGAATCGCAGAATCGTGGCTCTCGTTGGCTAAAAACGAGATGAGATAGGAGGGAAATCGAATTCCTCGGCCGCCATGGCGGCC 1376  
 GGGAGCATGCGACGTCGG 1394

**Figure 3-10. Partial genomic sequence of *Cmchi2*.** Introns are indicated by arrows.

**Table 3-3. Proteins with sequence identity to *Cmchi1* protein.** The nine highest scoring sequences from a BLAST search of the Swissprot database were plant chitinases with 54% to 85% sequence identity to the deduced *Cmchi1* protein.

	Accession	Source	Function	Score	Percent identity
1	P17541	<i>Cucumis sativus</i>	Acidic endochitinase precursor	521	85%
2	P29024	<i>Vigna angularis</i>	Acidic endochitinase precursor	361	62%
3	P36908	<i>Cicer arietinum</i>	Acidic endochitinase precursor	347	60%
4	P29060	<i>Nicotiana tabacum</i>	Acidic endochitinase precursor	346	61%
5	P36910	<i>Beta vulgaris</i>	Acidic endochitinase se2 precursor	341	60%
6	P23472	<i>Hevea brasiliensis</i>	Hevamine A	322	56%
7	P51614	<i>Vitis vinifera</i>	Acidic endochitinase precursor	316	56%
8	P19172	<i>Arabidopsis thaliana</i>	Acidic endochitinase precursor	312	54%
9	P29061	<i>Nicotiana tabacum</i>	Basic endochitinase precursor	300	54%
10	P49347	<i>Canavalia ensiformis</i>	Concanavalin B precursor	171	38%
11	P29025	<i>Rhizopus niveus</i>	Chitinase 1 precursor	150	34%
12	P29026	<i>Rhizopus microsporus</i> <i>var. oligosporus</i>	Chitinase 1 precursor	149	34%
13	P29027	<i>Rhizopus microsporus</i> <i>var. oligosporus</i>	Chitinase 2 precursor	144	34%
14	P46876	<i>Candida albicans</i>	Chitinase 1 precursor	131	30%
15	P29029	<i>Saccharomyces cerevisiae</i>	Endochitinase precursor	129	32%
16	P54197	<i>Coccidioides immitis</i>	Endochitinase 2 precursor	116	25%
17	P40953	<i>Candida albicans</i>	Chitinase 2 precursor	113	31%
18	P40954	<i>Candida albicans</i>	Chitinase 3 precursor	113	30%

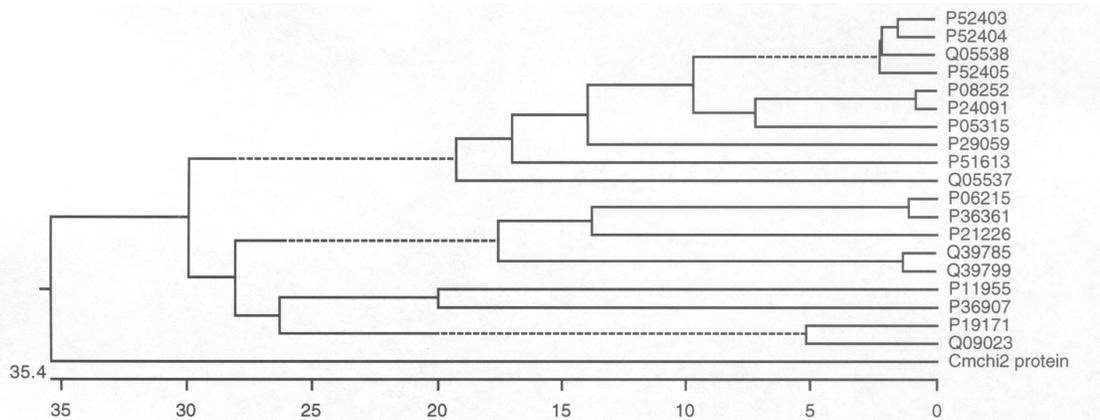


**Figure 3-11. Phylogenetic tree of *Cmchi1* protein with other related chitinases from databases.** The sequences from the proteins listed in Table 3 were aligned by the CLUSTAL method, and phylogenetic trees were generated for each protein. *Cmchi1* protein groups closely with class III chitinase from cucumber.



**Table 3-4. Proteins with sequence similarity to *Cmchi2* protein.** The 19 highest scoring sequences from a BLAST search of the Swissprot database were plant chitinases with 57% to 60% sequence identity to the deduced *Cmchi2* protein.

	Accession	Source	Function	Score	Percent identity
1	Q09023	<i>Brassica napus</i>	Endochitinase ch25 precursor	326	58%
2	P19171	<i>Arabidopsis thaliana</i>	Basic endochitinase precursor	321	57%
3	P24091	<i>Nicotiana tabacum</i>	Endochitinase B precursor	319	56%
4	P06215	<i>Phaseolus vulgaris</i>	Endochitinase precursor	319	56%
5	P51613	<i>Vitis vinifera</i>	Basic endochitinase precursor	319	58%
6	P08252	<i>Nicotiana tabacum</i>	Endochitinase A precursor	317	57%
7	P21226	<i>Pisum sativum</i>	Endochitinase A2 precursor	317	57%
8	P36361	<i>Phaseolus vulgaris</i>	Endochitinase ch5B precursor	317	56%
9	Q39799	<i>Gossypium hirsutum</i>	Endochitinase 1 precursor	315	60%
10	P05315	<i>Solanum tuberosum</i>	Endochitinase precursor	314	55%
11	P29059	<i>Nicotiana tabacum</i>	Endochitinase 3 precursor	312	58%
12	Q05538	<i>Lycopersicon esculentum</i>	Basic 30 KD endochitinase precursor	312	58%
13	Q39785	<i>Gossypium hirsutum</i>	Endochitinase 2 precursor	310	59%
14	P36907	<i>Pisum sativum</i>	Endochitinase precursor	309	59%
15	P52403	<i>Solanum tuberosum</i>	Endochitinase 1 precursor	306	57%
16	P52405	<i>Solanum tuberosum</i>	Endochitinase 3 precursor	305	57%
17	P52404	<i>Solanum tuberosum</i>	Endochitinase 2 precursor	305	56%
18	P11955	<i>Hordeum vulgare</i>	26KD Endochitinase 1 precursor	303	56%
19	Q05537	<i>Lycopersicon esculentum</i>	Basic endochitinase	302	55%



**Figure 3-13. Phylogenetic tree of *Cmchi2* protein and other related chitinases from databases.** The sequences from the proteins listed in Table 4 were aligned by the CLUSTAL method, and phylogenetic trees were generated for each protein. *Cmchi2* protein does not group closely with other related chitinases.

```

1  MKLYSLLSLLFLGLYLRSEGYASSSSND DYGRLINYYTTFNAMFKYQKDPQC Cucumis melo
1  .RSLAVVWVAVVAT.AMAI--GTAR--GS.SSIVSRAQ.DR.LLHRN.GA.Hordeum vulgare
1  .-.-.-.-.-Y.-CC.FLLF--LTG.FAQ...TIYTSDL..E.L.NRN.DR.Lycopersicon esculentum
1  .EFSGSPMA..CC.FFLF--LTG.LAQGI..SIYTSDL..E.L.NRN.GR.Nicotiana tabacum
1  .FMG SYLA..SF.VFLF--LTGTLAQN..SIYTSDL.DQ.L.NRN.AR.Petunia x hybrida
1  .R.LL--VLAW.SVLCCLKC--LAQ--...S...KNL.ERI.LHRN.AA.Solanum tuberosum

51  PSQGFYSYQAFLLTAARS.FGKLG.FATTGK.LATRKRRELLAFLAQTSHQTGG Cucumis melo
47  QAK...T.D..VA..AA.P--..G...SADAQ...YA...E...Hordeum vulgare
41  .AK...T.D..IA..N..P--..G...DDTA..K..IA..FG...E...Lycopersicon esculentum
49  .AN...T.D..IA..N..P--..G...DDTA..R..K..IA..FG...E...Nicotiana tabacum
48  FAYR...T.D..IA..N..P--..G...DDTA..K..IA..FG...E...Petunia x hybrida
44  AAK...T.E..I..TK..A--A.G...DTN...K..IA...E...Solanum tuberosum

181 WLTAPDGGPLFVGYCHIRESTE-DSYCKADPKWPCAKGQKY YGRGPMQLKG Cucumis melo
95  .A...A.FA...FKQ.RGASSD..TPSAQ...P.KR...I..SH.Hordeum vulgare
89  -SLSA...FAG...FV...GNQMG...-G.F...I..T.Lycopersicon esculentum
97  -SLSAE...FTG...FV.QNDQ-SD...-R...I..TN.Nicotiana tabacum
98  -TLS...YAG...FL...GNQMGN...-G...I..T.Petunia x hybrida
92  .A...Y.S...FKQ.QGSPGD..V.SQQ...P.K..F...I..ISY.Solanum tuberosum

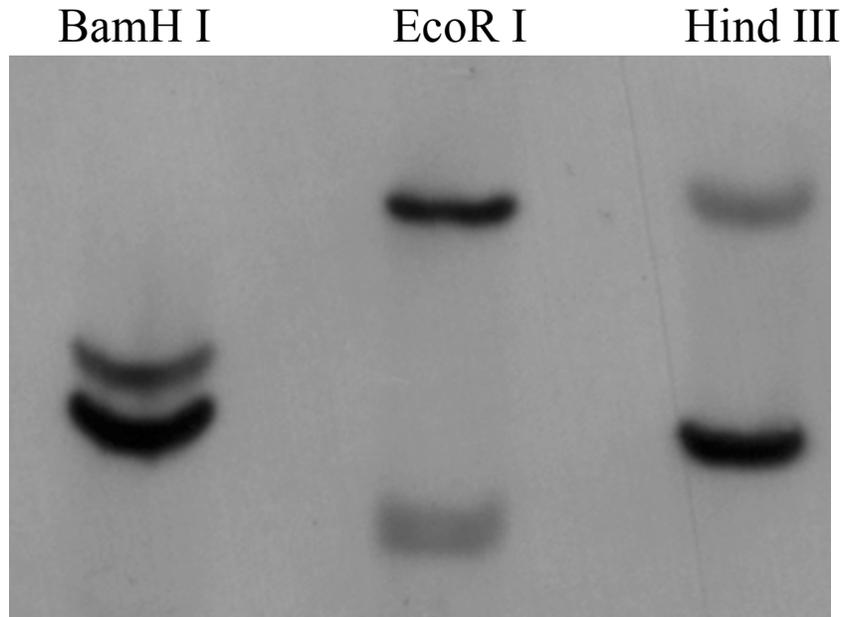
150 NQHYGQAGKALGLDLLKHPDLVAKDPYYSFKTAIWFVMTAQGIKPSCHDY Cucumis melo
145  .Y...P..R..I..V...A...T..AT..G...P.P...S..A.Hordeum vulgare
124 QS...DL..Q..I..Q..YN...T..AT...N...Lycopersicon esculentum
130 RN...EK...T..I..QE..VN...T..ATI...P..DN...S...Nicotiana tabacum
131 QS...DL..I..EQ..YN...T..AT...P..N...Petunia x hybrida
142 .Y...P..R..I..V..N...N...A...L...P..Q.P...A...Solanum tuberosum

200 MVGKWKPT EADKAAKRYPGYGVVSNII-GGSECGSGANTDYADRFGFYVR Cucumis melo
195 IA.Q.S.SG..R..G...F..IT...N..I...H.QDSR...I...K.Hordeum vulgare
174 IT.Q.T.SA.IAS.N.Q...IT...N..I...K.Q.P.QE...I...R.Lycopersicon esculentum
180 II.R.T.SA.Q.N...IT...N..I...I.R.DA.E..I..Y..R.Nicotiana tabacum
181 IT.R.T.SA.TS.N...IT...N..I...K.Q.AR.E..I..Y..R.Petunia x hybrida
192 IT.R.S.SA.S.G...F..IT...N..I...SK.S.AQ.MDN..I...R.Solanum tuberosum

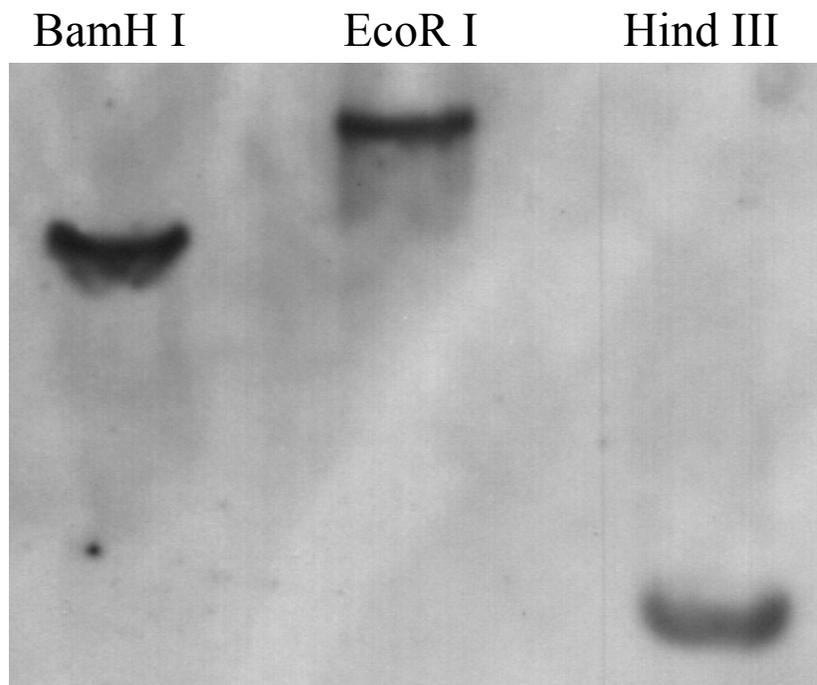
240 YCKMLGVNPGKHLDCFFQQPFRM Cucumis melo
245 .DI...GY..NN...YS..R..A.Hordeum vulgare
224 .TI..N.A..DN...YD..RN.AEA.Lycopersicon esculentum
230 .G...N.A..EN...YN..RN.GQG.Nicotiana tabacum
231 NYSIMN.A..DN...YN..RN.AEY.Petunia x hybrida
242 .QI...D..NN...AN..R..GQ.Solanum tuberosum

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**Figure 3-14. Amino acid sequence alignment of *Cmchi2* against other known class II chitinases from *Hordeum vulgare* (Leah et al., 1991), *Lycopersicon esculentum* (Danhash et al., 1993), *Nicotiana tabacum* (Payne et al., 1990), *Petunia x hybrida* (Linthorst et al., 1990), and *Solanum tuberosum* (Wemmer et al., 1994). Shown in dots (.) are identical amino acids while dashes (-) indicates gaps introduced to maximize alignment.**



**Figure 3-15. Southern blot of *Cmchi1* encoding class III chitinase in germinated muskmelon seeds.** Genomic DNA isolated from leaves of young muskmelon seedlings (20  $\mu$ g/lane) was digested with restriction enzymes BamH I , EcoR I and Hind III for 3 h. The digested DNA fragments were subjected to electrophoresis through 1.0% agarose gel and denatured. The denatured gel was transferred to a membrane. The membrane was probed with DIG-labeled *Cmchi1*cDNA.



**Figure 3-16. Southern blot of *Cmchi2* encoding class II chitinase in germinated muskmelon seeds.** Genomic DNA isolated from leaves of young muskmelon seedlings (20  $\mu$ g/lane) was digested with restriction enzyme BamH I, EcoR I and Hind III for 3 h. The digested DNA fragments were subjected to electrophoresis through 1.0% agarose gel and denatured. The denatured gel was transferred to a membrane. The membrane was probed with DIG-labeled *Cmchi2* cDNA.

## CHAPTER FOUR

### CHARACTERIZATION OF *Cmchi1* AND *Cmchi2* EXPRESSION IN GERMINATED MUSKMELON SEEDS AND EXPRESSION OF *Cmchi1* IN *E. COLI*

#### Materials and Methods

##### Plant Material

Muskmelon (*Cucumis melo* cv. Top Mark) seeds were decoated and germinated at 30°C in the dark on Petri dishes on three layers of germination blotter papers (Anchor Paper Co.) moistened with distilled water. In some cases, germinated seeds were dissected to separate the radicles from the rest of the seeds. In another case, root, stem, leaf, and flower tissue were also collected from mature muskmelon plants growing in the field, frozen in liquid nitrogen, and stored in - 80 °C.

##### Treatment of Muskmelon Seeds with 0.3 mM BTH or 1 mM SA

Muskmelon seeds were imbibed on germination paper moistened with distilled water for 8, 16, or 24 h (control) or imbibed on the germination paper moistened with 0.3

mM benzothiadiazole (BTH) or 1 mM salicylic acid (SA) for 8, 16 or 24 h (BTH and SA treatments) at 30°C in the dark. After each imbibition time, seeds were removed and stored at -80 °C for later RNA extraction.

#### Total RNA isolation

Total RNA extraction was conducted as described in Chapter Three. RNA was extracted from seeds imbibed with water for 2, 4, 8, 12, 16, 20, 24, 36 or 48 h. RNA was also isolated from imbibed seeds with BTH or SA treatment as described above. RNA extraction was also conducted for roots, stems, leaves, and flowers of mature muskmelon plants growing in the field.

#### Poly (A<sup>+</sup>) RNA isolation

Poly (A<sup>+</sup>) RNA was isolated from total RNA using Oligotex mRNA Spin-Columns (QIAGEN) according to the manufacturer's protocol. Briefly, 500 µg total RNA in a final volume of 500 µL was mixed with 500 µL OBB buffer (10 mM Tris-HCl, pH 7.5, 1M NaCl, 2 mM EDTA, and 0.2% SDS, supplied in the Kit), and 55 µL Oligotex Suspension (Oligotex particles in 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 0.1% SDS, and 0.1% sodium azide, supplied in the Kit), and incubated at 70°C for 3 min. The sample was incubated at room temperature for 10 min to allow hybridization between the oligo dT<sub>30</sub> of the Oligotex particle and the Poly (A<sup>+</sup>) tail of the mRNA. The Oligotex: mRNA complex was then pelleted by centrifugation for 2 min at maximum speed (14,000 rpm). The pelleted Oligotex: mRNA complex was mixed with 400 µL

OW2 buffer (10 mM Tris-HCl, pH 7.5, 150 M NaCl, and 1 mM EDTA, offered in the Kit) and then transferred to the spin column. After centrifugation, the column was washed again with 400 $\mu$ L OW2 buffer, and the mRNA was eluted from the column with hot OEB buffer (5 mM Tris-HCl, pH 7.5 at 70°C, supplied in the Kit) by centrifugation.

#### RNA Probe Template Preparation

RNA probe template preparation involved several steps. First, a fragment for the synthesis of the RNA probe was prepared by PCR using the vector containing the full length of either *Cmchi1* or *Cmchi2* cDNA as template. A fragment was amplified from *Cmchi1* cDNA using primer 48EM (5'-GTC AAA GTC CTC CTC TCT ATC G-3') and 48RV (5'-AGC TTA ATT CAG CCG ATG CTG -3'). The primers used to amplify a fragment from *Cmchi2* cDNA were 24 DM (5'-ATG GTC GTG GAC CAA TGC AA-3') and 24 RV (5'-TCC CTC CTA TCT CAT CTC GT-3'). Second, the resulting PCR products, from *Cmchi1* with a size of 598 bp or from *Cmchi2* with a size of 527 bp, were then purified in a 1.0% agarose gel. Third, the gel-purified fragments were ligated to a T7 adapter (T7 promoter, Ambion). Finally, the ligation mixtures were used as the templates for PCR amplification using the T7 adapter primer 1 (supplied in the kit, Ambion) and a gene specific primer 48EM from *Cmchi1* and 24 DM from *Cmchi2*. The resulting PCR products with the T7 promoter at their 3' ends have an additional 62 bp as compared to the fragments without the T7 promoter. These PCR products were purified through QIAGEN spin columns and used as the templates for synthesis of RNA probes.

### RNA Probe Synthesis

DIG-labeled RNA probes were synthesized using the DIG RNA Labeling Kit (SP6/T7) according to the manufacturer's protocol (Boehringer Mannheim). The transcription mix contained RNA template as described above (1  $\mu$ g), 10X transcription (2  $\mu$ L), NTP labeling mixture (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 6.5 mM dUTP, and 3.5 mM DIG-11-dUTP in Tris-HCl, PH 7.5, 2  $\mu$ L), T7 RNA polymerase (2  $\mu$ L), RNase inhibitor (1  $\mu$ L) and DEPC-treated water. The transcription reaction was carried out for 2 h at 37 °C and terminated by the addition of 2  $\mu$ L 0.2 mM EDTA. The synthesized RNA probe was then stored at -80°C prior to hybridization.

### RNA Gel Blot Hybridization

Total RNA was extracted from seeds or seed tissues of germinating or germinated seeds as described above. Poly (A<sup>+</sup>) RNA was purified, and 1.75  $\mu$ g to 5  $\mu$ g were subjected to electrophoresis on a 1% agarose gel containing 1X denaturing gel buffer (supplied in the NorthernMax kit, Ambion) for 2 h at 70 V. The RNA was transferred to a positively charged membrane (Hybond-N<sup>+</sup>, Amersham Pharmacia Biotech) by a downward transfer system and then UV-cross-linked. The RNA blotted membrane was then prehybridized with 10 mL Ultrahy buffer (Ambion) for 1 h and hybridized with DIG-labeled RNA probe (0.1 nM) overnight at 68°C. The membrane was washed at room temperature twice in a low stringency solution (e.g., equivalent to 2x SSC + 0.1% SDS, 5 min, #1 washing solution in NorthernMax kit, Ambion) and twice at 68°C in high

stringency solution (e.g., equivalent to 0.1X SSC + 0.1% SDS, 20 min, #2 washing solution NorthernMax kit, Ambion). The hybridization signal was detected using the chemiluminescent system as described in Chapter III.

#### Construction of an Expression Vector to Express *Cmchi1* Fusion Protein in *E.coli*.

The coding region (without signal peptide) of the *Cmchi1* cDNA was amplified by PCR using a BamH I site-linked forward primer (5'-**CGGGATCC**GCTGGAATCGCCATC-3') and a Xba I site-linked reverse primer (5'-**CGTCTAGAT**CAGCCGATGCTGCC-3'). Nucleotides in bold are the restriction site of BamH I and Xba I, respectively. The product was digested with BamH I and Xba I and ligated into the BamH I and Xba I sites of the maltose-binding protein (MBP) expression vector pMAL-c2x (New England Biolabs, Inc., Beverly, MA). The empty vector and the vector with insert were transformed into competent cells of a proteinase-deficient strain (BL21) of *E. coli*. Transformed cells were selected using blue-white screening with IPTG-Xgal as described in Chapter III.

#### Pilot Experiment to Determine the Localization of the *Cmchi1* Fusion Protein

A white colony expected to contain the recombinant plasmid was allowed to grow in 2 mL LB + ampicillin (100 µg/mL) overnight with shaking at 250 rpm. 40 mL rich broth + glucose and ampicillin (Manual from the New England Biolabs) was inoculated with 0.4 mL of an overnight culture of cells containing the *Cmchi1*-MBP fusion plasmid. The culture was grown at 37°C with shaking at 250 rpm until the cell

concentration reached  $2 \times 10^8$  cells/mL ( $A_{600}$  of  $\sim 0.5$ ). For the non-induced sample, 1 mL culture was removed from the 40.4 mL culture and pelleted by centrifugation at maximum speed in an Eppendorf centrifuge. The supernatant was discarded and the cell pellet was resuspended in 50  $\mu$ L SDS-PAGE sample buffer (20% bromophenol blue, 20% glycerol, 4% SDS, 10%  $\beta$ -mercaptoethanol, 25% Upper Tris). After vortexing, the sample was stored at  $-20^\circ\text{C}$ .

IPTG was added to the remaining culture to yield a final concentration of 0.3 mM. After 2 h of growth at  $37^\circ\text{C}$  with shaking at 250 rpm, a 0.5 mL aliquot was removed and cells were pelleted and saved as the non-induced sample.

Cells in the remaining culture were harvested by centrifugation at 4,000  $g$  for 10 min. The supernatant was discarded and the cell pellet was resuspended in 5 mL column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA). The resuspended cells were subject to a freeze-thaw cycle to lyse cells by freezing in a dry ice-ethanol bath for 20 min and thawing in cold water. The thawed cells were placed in an ice-water bath and subjected to sonication consisting of 6 x 15 seconds with 30 seconds pauses at setting 2, Branson Sonifier 450 (Fisher). Sonicated cells were clarified by centrifugation at 9,000  $g$  at  $4^\circ\text{C}$  for 20 min. The supernatant was saved as the crude soluble fraction and the pellet was resuspended in 5 mL column buffer for analysis of insoluble protein (i.e., those proteins in inclusion bodies). A 5  $\mu$ L aliquot of each fraction was suspended in an equal volume of 2 X SDS-PAGE sample buffer and stored at  $-20^\circ\text{C}$ .

### SDS-PAGE for *Cmchi1* Recombinant Protein

SDS-PAGE was performed to analyze samples saved in the pilot experiment according to method of Laemmli (1970) in Tris-Glycine buffer (pH 8.0) with a 12% separating gel (pH 8.8) and 4% stacking gel (pH 6.8). After electrophoresis, the protein was stained with 0.1% Coomassie brilliant blue R-250 in a solution of methanol: water: acetic acid (40%, 10%) for 40 min. The gel was destained in a solution of methanol:acetic acid (40:10). The gel was dried between two cellophane sheets.

### Renaturation of *Cmchi1* Recombinant Protein

As the recombinant protein was present primarily in the insoluble fraction, the insoluble protein from the pilot experiment was renatured according to the method described by Gierlich et al. (1999). Briefly, the insoluble pellet was mixed with 2 mL 8 M urea in 0.1 M NaH<sub>2</sub>PO<sub>4</sub> / 0.01 M Tris, pH 8.7. Subsequently, 20 mL renaturing buffer (25 mM boric acid, 8 mM cysteine, 1 mM cystine, 5 mM EDTA, pH 8.7) were added. After incubation with gentle stirring overnight at 4 °C in the dark, the renatured protein was desalted by dialysis against 4 L column buffer 4 °C.

### Affinity Chromatograph Purification of *Cmchi1* Recombinant Protein

The pilot experiment was conducted to determine the purification efficiency of amylase resin. Amylase resin (200 µL, New England Biolabs) was placed in a microfuge tube and pelleted by brief centrifugation. The supernatant was discarded, and the resin was resuspended with 1.5 mL column buffer and followed by centrifugation. After

washing the resin twice with 1.5 mL column buffer, the resin was resuspended in 200  $\mu$ L column buffer. The renatured protein (50  $\mu$ L) was mixed with 50  $\mu$ L of the amylose resin slurry. The mixture was incubated on ice for 15 min and clarified by centrifugation. The supernatant was discarded, and the pellet was washed with 1 mL column buffer followed by microcentrifugation. Half of the resin was resuspended in 50  $\mu$ L SDS-PAGE sample buffer and stored at  $-20^{\circ}\text{C}$  for electrophoresis. The remaining resin was mixed with 50  $\mu$ L column buffer + 10 mM maltose followed by centrifugation. The supernatant was mixed with 50  $\mu$ L SDS-PAGE sample buffer and stored at  $-20^{\circ}\text{C}$  for electrophoresis.

The renatured protein was purified by affinity column chromatograph. Briefly, amylose resin (New England Biolabs) was poured into a 2.5 x 10 cm column and equilibrated with eight column volumes of column buffer. The renatured protein was loaded onto the column at a flow rate of 1 mL/min for a 2.5 cm column. The column was then washed with twelve column volumes of column buffer, and finally the fusion protein was eluted from the column with column buffer + 10 mM maltose. Fractions (3 mL each) were collected for analysis.

#### Cleavage of *Cmchi1* Recombinant Protein with Factor Xa

The purified protein was concentrated by ultrafiltration on a YM 10 membrane (Centricon 3, Amicon) to a final concentration of 3.6 mg/mL. A pilot experiment was conducted to evaluate Factor Xa mediated cleavage of the concentrated fusion protein.

5.6  $\mu\text{L}$  of fusion protein was mixed with 14.4  $\mu\text{L}$  column buffer and 1  $\mu\text{L}$  protease Factor Xa diluted to 200  $\mu\text{g}/\text{mL}$ , and the reaction was incubated at room temperature for 2, 4, 8 or 28 h. After each time period, 5  $\mu\text{L}$  of the cleavage reaction was removed, mixed with 5  $\mu\text{L}$  2X SDS-PAGE sample buffer, and stored at 4  $^{\circ}\text{C}$  for analysis.

### SDS-PAGE and Western blotting

Proteins isolated from the embryonic axis or the radicle tissues were separated by SDS-PAGE (12 % (w/v) acrylamide) using the buffer system of Laemmli (1970). The separated proteins were electrophoretically transferred to a PVDF membrane (Immobilon-P, Millipore) using a semidry transfer apparatus (Amersham-Pharmacia Biotech) according to the manufacturer's recommendations. The transfer buffer was 48 mM Tris, 39 mM Gly, pH 8.4, 1.3 mM SDS, 20% methanol. Blocking and incubation in primary and secondary antibodies were performed with "Blotto" (Johnson et al., 1984). The Blot was incubated in Blotto solution with the antibody raised against the class III chitinase in cucumber provided by Novartis (Research triangle park, Northern Carolina). The antibody was diluted 1:1000 with Blotto at room temperature. The Blot was rotated gently on a shaker for 2 h (primary) and 1 h (secondary). The blot was washed between steps using 200 mM NaCl buffered with 50 mM Tris-HCl, pH 7.4. Colorimetric detection with the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (both from Sigma) was catalyzed by alkaline phosphatase-conjugated, goat anti-rabbit antibody (KPL, Gaithersburg, MD).

## **Results**

To study expression patterns of both *Cmchi1* and *Cmchi2* during imbibition and after radicle emergence, total RNA was isolated from seeds imbibed for 2, 4, 8, 12, 16, 20, 24, 36 or 48 h in the dark at 30 °C, and Poly (A<sup>+</sup>) RNA was isolated from each sample for RNA gel blot analysis. In the dark at 30 °C, by definition, germination or radicle emergence of muskmelon seeds was first detectable after seeds imbibed for 16 h. *Cmchi1* mRNA was detected in seeds imbibed for at least 20 h, and the level increased with increasing imbibition time (Fig. 4- 1A). *Cmchi2* mRNA was detected in seeds imbibed for at least 16 h, and the level also increased with increasing imbibition time (Fig. 4-1B). Both *Cmchi1* and *Cmchi2* mRNA were not detected in seeds imbibed for less than 16 h or in seeds at 25 and 35 DAA (data not shown). Equal amounts of mRNA were loaded in each lane as determined by the  $\beta$ -Actin7 loading control (Fig. 4-1A and Fig. 4-1B, lower panel).

For the localization of both *Cmchi1* and *Cmchi2* mRNA, decoated muskmelon seeds were imbibed for 24 h, and the radicle tissue was dissected from the rest of decoated seeds. Poly (A<sup>+</sup>) RNA isolated from each tissue was subjected to Northern blot analysis. Both *Cmchi1* and *Cmchi2* mRNAs were present at relatively high levels in the radicle tissue as compared to the remaining seed tissue (Fig. 4-2). *Cmchi1* and *Cmchi2*

mRNA apparently were also detected in root tissue but not in leaf, stem, and flower tissue of mature muskmelon plants (Fig. 4-3).

SA and BTH have been shown to induce gene expression associated with systemic acquired resistance (SAR) in a number of studies. To determine the inducibility of *Cmchi1* and *Cmchi2*, muskmelon seeds were imbibed for 8, 16 or 24 h on germinated paper moistened with 1 mM SA or 0.3 mM BTH. RNA gel blots showed that, in muskmelon seeds imbibed in 1 mM SA for 8 h, the level of *Cmchi1* mRNA did not increase significantly. An obvious increase of *Cmchi1* mRNA level occurred in seeds imbibed in SA for 16 h; and, when seeds were imbibed in SA for 24 h, *Cmchi1* mRNA level increased dramatically (Fig. 4-4). When imbibed in 0.3 mM BTH, the level of *Cmchi1* mRNA slightly increased in seeds after 16 but not by 8 h. When seeds were imbibed in 1 mM SA or 0.3 mM BTH, the level of *Cmchi2* mRNA did not increase relevant to control at any time examined (Fig. 4-5). The expression of  $\beta$ -actin loading controls in Figure 4-5 and 4-6 slightly increased with in imbibition time.

To test the identity of the protein encoded by *Cmchi1*, *Cmchi1* cDNA was expressed in *E. coli*. As shown in Figure 4-6, as expected, IPTG induction of a 70 kDa protein was observed (Lane 3). Most of the fusion protein was found to be present in the insoluble fraction (Lane 5) and not in the crude soluble fraction (Lane 4). When protein extracted from the insoluble fraction was renatured, it could be recovered into a soluble

form (Lane 6). This soluble fusion protein bound specifically to amylose resin (Lane 7) and was eluted into maltose-containing buffer (Lane 7).

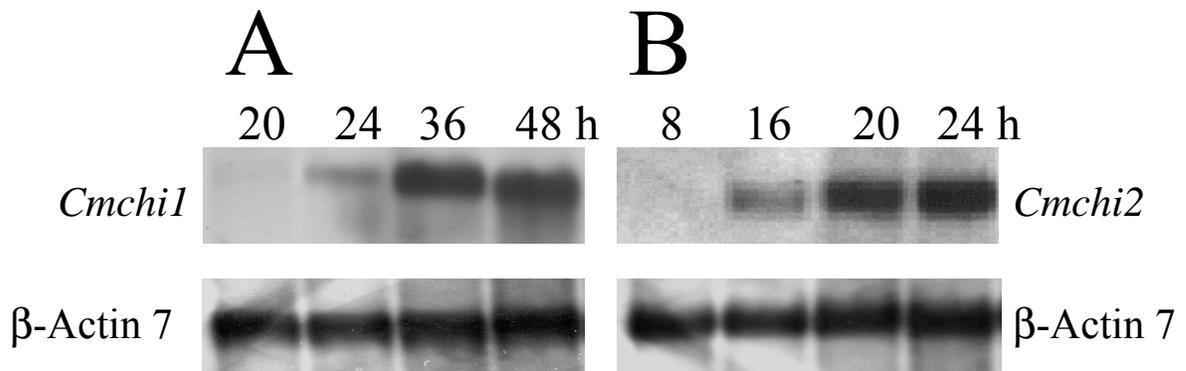
The degree of purity of the eluted fusion protein was further investigated. The eluted protein was subjected to SDS-PAGE along with various amounts of renatured protein prior to the purification step. As shown in Figure 4-7, when similar amounts of eluted protein and renatured protein without purification were compared, more than one band was detected in the crude renatured protein (Lane 3) but not in the affinity purified protein (Lane 2). This indicates that the fusion protein can specifically bind to amylose resin and can be eluted with maltose containing buffer.

The purified fusion protein was digested by protease Factor Xa, but the digestion was incomplete (Fig. 4-8). When the purified fusion protein was incubated with Factor Xa for 2, 4, 8 or 28 h (lane 3, 4, 5, 6), the degree of digestion increased only slightly as incubation time increased.

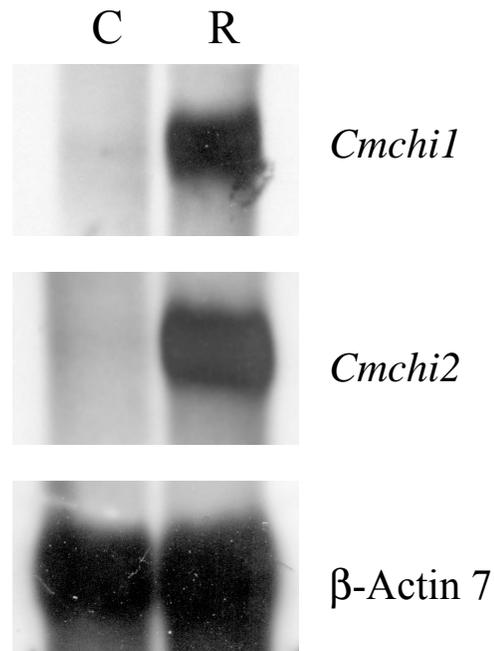
The size of cleaved *Cmchi1* protein was further determined by SDS-PAGE. Maltose binding protein, Factor Xa, the digested *Cmchi1* fusion protein and the undigested *Cmchi1* fusion protein were subjected to electrophoresis. As shown in Figure 4-9, the *Cmchi1* protein migrated on the gel to a position close to size marker 29 kDa (Lane 4). Undigested purified *Cmchi1* fusion protein showed a certain degree of degradation, because multiple bands were present, including the maltose binding protein (Lane 5).

In order to characterize the purified fusion protein, activity assays and western blotting were performed. A gel diffusion assay was performed to check the enzymatic activity. The purified expressed protein did not show any enzymatic activity (data not shown). Western blotting using an antibody raised against the class III chitinase protein purified from cucumber was also inconclusive as this antibody recognized the purified *Cmchi1* fusion protein and other unknown proteins isolated from the embryonic axis or the radicle tissue (data not shown).

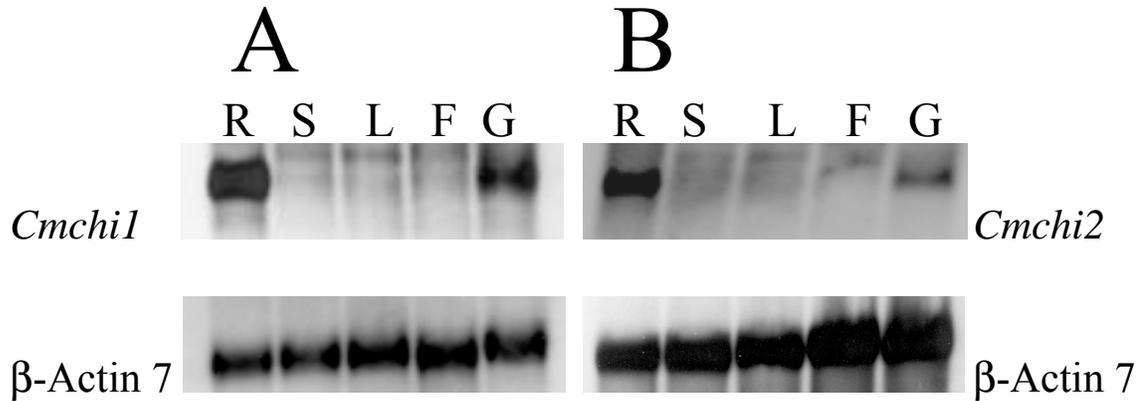
## **Figures**



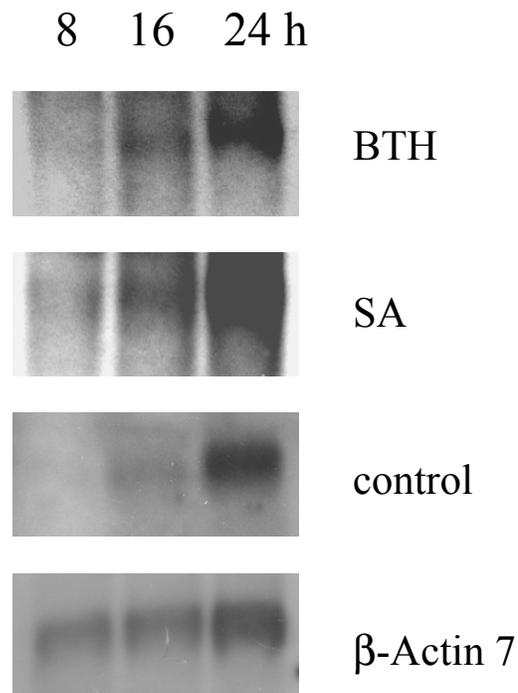
**Figure 4-1. Northern blot of analysis of *Cmchi1* (A) and *Cmchi2* (B) gene expression in muskmelon seeds during or after germination.** Poly (A<sup>+</sup>) RNA was isolated from seeds imbibed for 8, 16, 20, 24, 36 and 48 h. Poly (A<sup>+</sup>) RNA (2 μg per lane) was separated on a formaldehyde-containing agarose gel and transferred to a membrane. A DIG-labeled antisense *Cmchi1* or *Cmchi2* (including part of coding region and 3' UTR region) RNA was used to probe the membrane. Hybridization signal was detected by alkaline phosphatase catalyzed chemiluminescence. After detection, the same membrane was stripped and reprobed again with a DIG-labeled antisense β-Actin 7 probe made from *Arabidopsis thaliana* gene ACT7 (shown in lower panel).



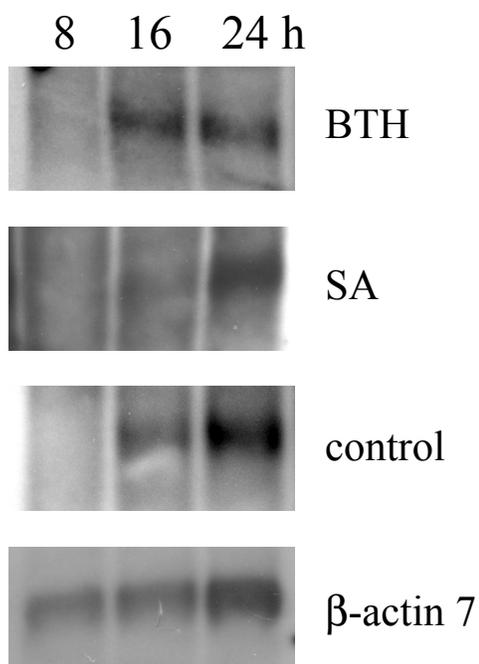
**Figure 4-2. Northern blot analysis of both *Cmchi1* and *Cmchi2* gene expression in radicle tissue, the cotyledon and endosperm.** Muskmelon seeds were imbibed for 24 h and dissected into the radicle tissue (R) and the cotyledons including endosperm tissue (C). Poly (A<sup>+</sup>) RNA was isolated from each tissue fraction. Poly (A<sup>+</sup>) RNA (2 µg per lane) was separated on a formaldehyde-containing agarose gel and transferred to a membrane. A DIG-labeled antisense *Cmchi1* or *Cmchi2* (including part of coding region and 3' UTR region) RNA was used to probe the membrane. Hybridization signal was detected as for Fig. 4-1. Stripping and reprobing the membrane with β-Act7 was as described for Fig. 4-1.



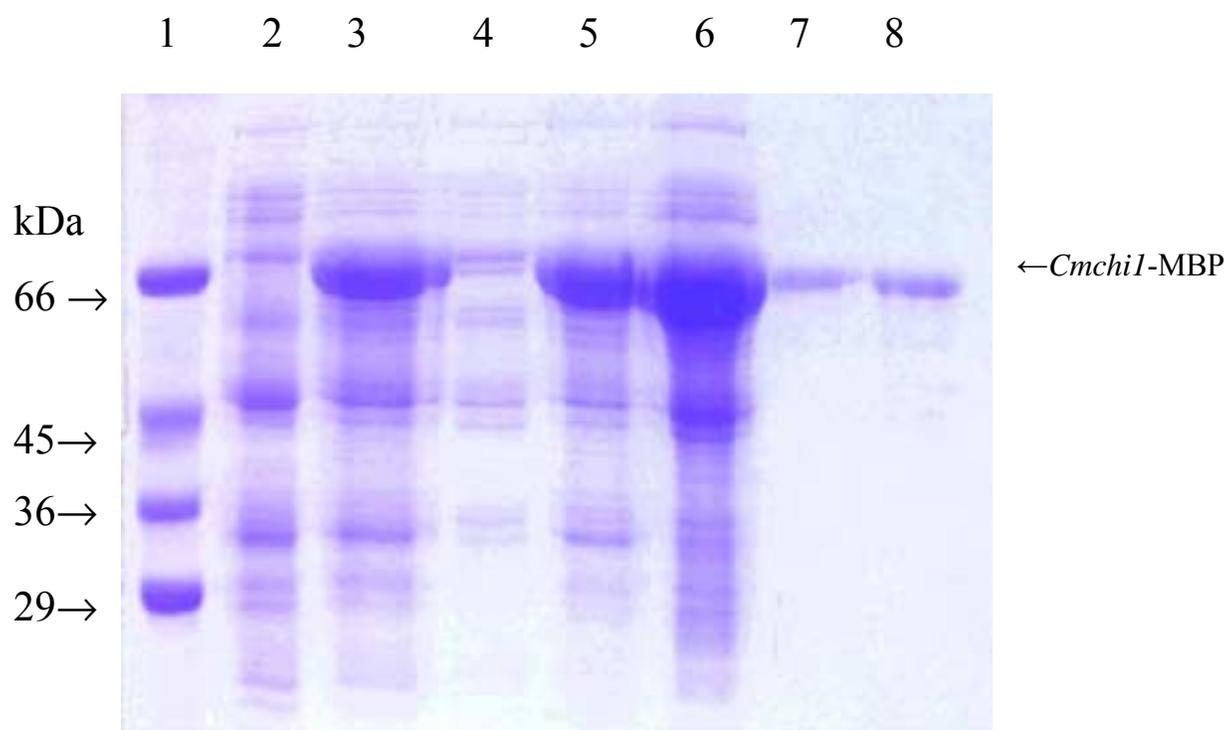
**Figure 4-3. Northern blot analysis of *Cmchi1* or *Cmchi2* gene expression in different tissues and germinated muskmelon seeds.** Poly (A<sup>+</sup>) RNA was isolated from roots (R), stems (S), leaves (L), and flowers (F) of mature muskmelon plants growing in the field and seeds imbibed for 24 h (G). Poly (A<sup>+</sup>) RNA (1.75  $\mu$ g per lane) was separated on formaldehyde-containing agarose gels and transferred to membranes. A DIG-labeled antisense *Cmchi1*(A) or *Cmchi2*(B) (including both part of coding region and 3' UTR region) RNA was used to probe the membrane. Hybridization signal was detected as for Fig. 4-1. Stripping and reprobing the membrane with  $\beta$ -Act7 was also conducted as for Fig. 4-1.



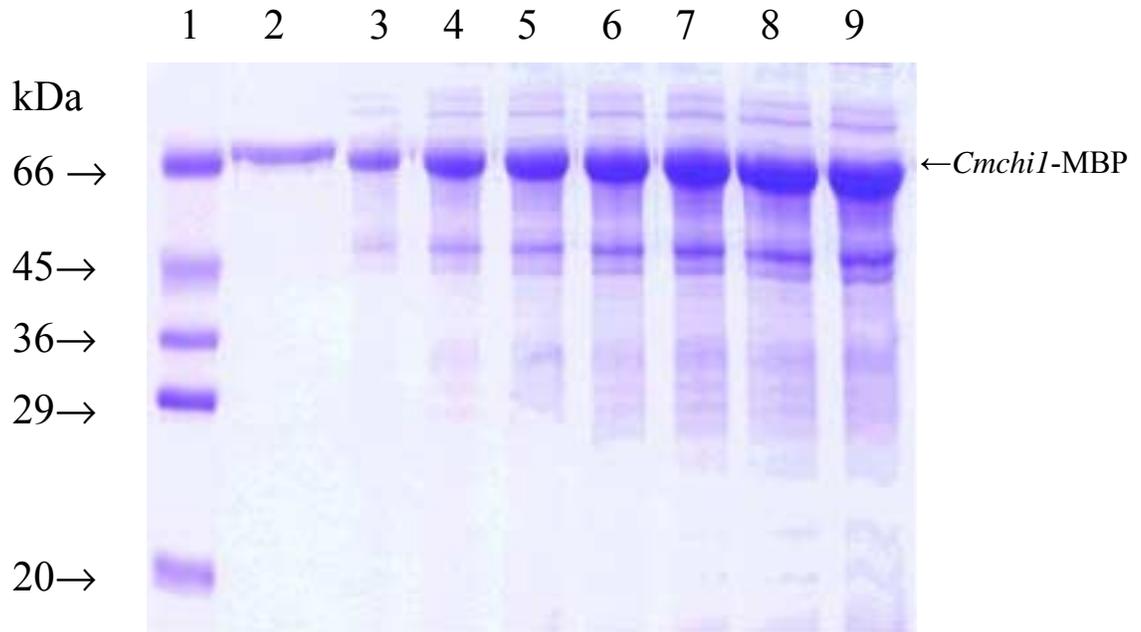
**Figure 4-4. Northern blot analysis of *Cmchi1* gene expression in muskmelon seeds imbibed in water (control) and BTH or SA (treatment).** Poly (A<sup>+</sup>) RNA was isolated from muskmelon seeds imbibed in water for 8, 16 or 24 h (Control) or from seeds imbibed on the germination paper moistened with 0.3 mM BTH or 1 mM SA for 8, 16 or 24 h (BTH, SA). Poly (A<sup>+</sup>) RNA (2  $\mu$ g /lane) was separated on a formaldehyde-containing agarose gel and transferred to membranes. A DIG-labeled antisense *Cmchi1* (including part of coding region and 3' UTR region) RNA was used to probe the membrane. Hybridization signal was detected as for Fig. 4-1. Stripping and reprobing the membrane with  $\beta$ -Act7 was also conducted as for Fig. 4-1.



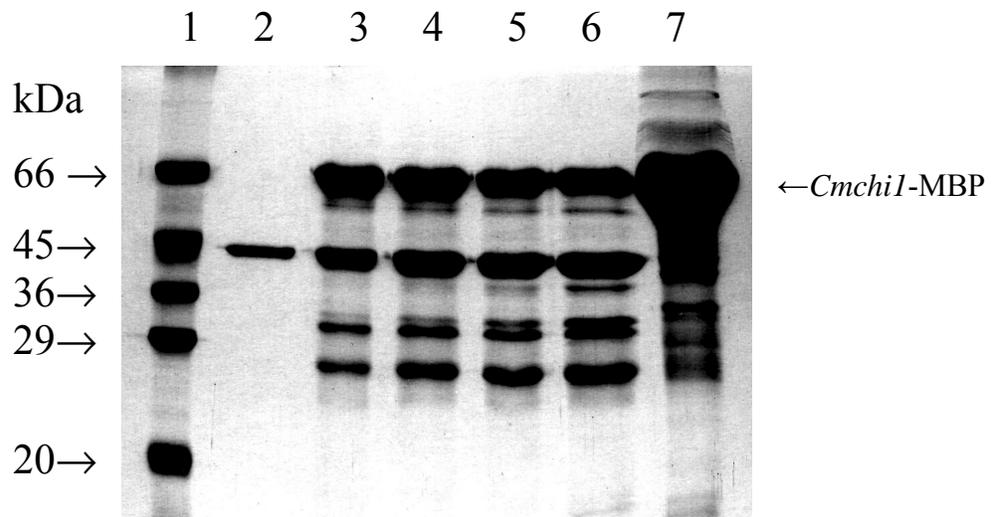
**Figure 4-5. Northern blot analysis of *Cmchi2* gene expression in muskmelon seeds imbibed in water (control) and BTH or SA (treatment).** Poly (A<sup>+</sup>) RNA was isolated from muskmelon seeds imbibed in water for 8, 16 or 24 h (Control) or from seeds imbibed on the germination paper moistened with 0.3 mM BTH or 1 mM SA for 8, 16 or 24 h (BTH, SA). Poly (A<sup>+</sup>) RNA (2 μg / lane) was separated on a formaldehyde-containing agarose gel and transferred to membranes. A DIG-labeled antisense *Cmchi2* (including part of coding region and 3' UTR region) RNA probe was used to probe the membrane. Hybridization signal was detected as for Fig. 4-1. Stripping and reprobing the membrane with β-Act7 was also conducted as for Fig. 4-1.



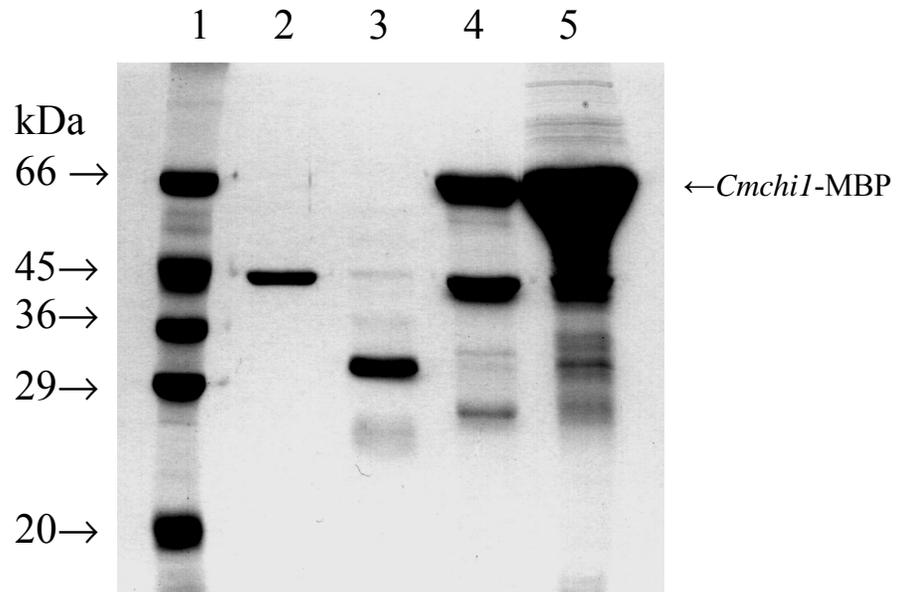
**Figure 4-6. SDS-PAGE of *Cmchi1* fusion protein samples prepared in the pilot experiment and proteins resulting from renaturing and purification steps.** Lane 1) molecular mass standard, lane 2) protein from the uninduced cells, lane 3) protein from the induced cells, lane 4) protein from the crude extract after sonication, lane 5) protein from the insoluble fraction, lane 6) renatured protein from the insoluble fraction, lane 7) bound fraction, lane 8) protein eluted from the amylose resin with column buffer + 10 mM maltose. Arrow indicates the position of the *Cmchi1*-MBP fusion protein.



**Figure 4-7. SDS-PAGE of the purified fusion protein and renatured fusion protein prior to purification.** Lane 1) protein size marker, lane 2) fusion protein purified using the amylose resin, lanes 3 to 9 including the level of concentrated renatured protein prior to purification ranging from 1  $\mu$ L to 7  $\mu$ L. The arrow indicates the position of the *Cmchi1*-MBP fusion protein.



**Figure 4-8. SDS-PAGE of the fusion protein samples cleaved by Factor Xa.** Shown in lane 1 are the molecular mass standard. Lane 2 is maltose-binding protein provided with the kit. Lanes 3, 4, 5, 6, and 7 are protein samples taken from the cleavage reaction after 2, 4, 8 and 28 h, respectively. Lane 7 is the purified uncut fusion protein. The arrow indicates the position of the *Cmchi1*-MBP fusion protein.



**Figure 4-9. SDS-PAGE of protein samples from different sources.** Lane 1) molecular mass standard , lane 2) maltose-binding protein, lane 3) Factor Xa, lane 4) protein samples taken from the digestion reaction catalyzed by Factor Xa, lane 5) purified uncut fusion protein. The arrow indicates the position of the *Cmchi1*-MBP fusion protein.

## Discussion

Gel diffusion assay is a simple and easy way to detect chitinase activity.

To test for chitinase activity in muskmelon seeds, a simple assay was developed. This new assay combined both the gel-diffusion assay principle used to detect other hydrolases (Dingle et al., 1953; Wood et al., 1988; Downie et al., 1994) and the method used to detect chitinase after polyacrylamide gel electrophoresis (Trudel et al., 1989; Pan et al., 1991). This new assay uses a soluble modified form of substrate, glycol chitin (Koga et al., 1983; Boller et al., 1983; Pan et al., 1991; Trudel et al., 1989;) and the affinity of calcofluor for chitin (Maeda et al., 1967). The ability of calcofluor to fluoresce when bound to undigested chitin simplified the detection of chitinase activity in the presence of UV light. Compared to other previously established assays for chitinases such as the viscosimetric assay (Otakara, 1961), colorimetric assay (Boller, et al., 1983) and radiometric assay (Molano et al., 1977), the gel diffusion assay for chitinase is simple, fast, and easy to perform. The results are quantitative and easy to visualize. Two controls (both the incubation buffer and the boiled crude protein extract) did not show any enzymatic activity. The addition of standard chitinase produced diffusion zones in the gel plate, and the area of diffusion zone was proportional to the units of standard enzyme applied (Fig. 2-1). The susceptibility of glycol chitin to cleavage catalyzed by other non-chitinase hydrolases present in muskmelon seed protein extracts was not tested,

however, as glycol chitin is a well-established standard substrate for testing chitinase activity in different assays (Boller et al., 1983; Trudel et al., 1989; Pan et al., 1991).

#### Multiple chitinase isoforms are developmentally regulated in muskmelon seeds

Chitinase activity was detected in developing muskmelon seeds at least 40 DAA and in fresh mature seeds (Fig. 2-2). One acidic chitinase isoform (AD1) and three basic isoforms (BD1, BD2, BD3) were present in developing seeds and mature seeds. No enzyme activity was detected in seeds before 40 DAA. Both the gel diffusion assay (Fig. 2-2) and native PAGE experiments (Fig. 2-3, 2-4) revealed that chitinase activity was detectable in seeds at 40 DAA, indicating that chitinase isoforms are developmentally regulated. Chitinase has been reported to be developmentally regulated in other seeds. In soybean, a gene encoding a class III endochitinase was expressed only in seeds from the early stage until the late middle stage of development, but it was not expressed in any other tissues (Yeboah et al., 1997). In carrot, the EP3 chitinase protein was detected in the integument at 10 days after pollination (DAP) and in the developing endosperm at 20 DAP (van Hengel et al., 1998). In lupine, a class III chitinase gene, IF3, was also expressed during seed development (Regalado et al., 2000).

The presence of chitinase in developing seeds suggests that chitinase may act as a preformed defense mechanism to prevent possible pathogenic attack. A similar hypothesis has also been proposed for the function of chitinase present in cereal grains, where chitinase may act as a part of a preemptive strategy to protect seeds against

microbial attack (Fincher, 1989). One study suggested that chitinase might be a storage protein in banana (Clendennen et al., 1998). In soybean seeds, endochitinase expression during the early stages of development appeared remarkably higher than that of storage proteins such as glycinin at the same stage, suggesting that there is a need to protect the seed against potential pathogen attack at its most vulnerable stages (Yeboah et al., 1998). In our study, northern blot analysis showed that the two genes cloned from muskmelon seeds after radicle emergence were expressed not in developing seeds but only after radicle emergence. Other chitinase genes, encoding these chitinases present in developing muskmelon seeds, must therefore exist, and their gene products may contribute to seed protection during seed development.

Chitinases present in developing seeds may also be involved in embryogenesis. De Jong et al. (1992) showed that a 32-kDa acidic endochitinase, EP3, was capable of rescuing somatic embryos in the mutant carrot cell line ts11 that was unable to form somatic embryos at the non-permissive temperature of 32° C. Later studies showed that this endochitinase was present in developing carrot seeds in the integument at 10 days after pollination (DAP) and in the developing endosperm at 20 DAP, suggesting that chitinase may be involved in zygotic embryogenesis (van Hengel et al., 1998). However, our study did not investigate this hypothesis, as we did not study the chitinase during early embryogenesis.

Multiple chitinase isoforms are present in imbibed seeds and in seeds after radicle emergence, and they are regulated in a tissue-specific manner.

Multiple chitinase isoforms were present in seeds during imbibition and after radicle emergence, and they were present in both endosperm and embryo tissues. Acidic chitinase activity was detected only in the endosperm tissue not in embryo tissue, indicating chitinase was regulated in a tissue-specific manner. The acidic chitinase isoform (AE1) persisted in endosperm tissue during imbibition and after radicle emergence (Fig. 2-5). A new acidic chitinase isoform (AE2) accumulated in seeds after the start of radicle emergence (Fig. 2-5). It is possible that this isoform was newly synthesized in seeds only after radicle emergence. It is also possible that this isoform is a modified form of the AE1 acidic isoform. Some studies have shown that some chitinases can be modified through glycosylation. In sugar beet, a class IV chitinase is apparently glycosylated with xylose (Nielsen et al., 1994).

The BR3 isoform was detected in the embryonic axis tissue at 4 h after the start of imbibition, barely detected at 12 and 24 h, but increased in the radicle tissue after radicle emergence (Fig. 2-10). It is possible that activity is not significant during imbibition, and after radicle emergence, the expression of this gene may be induced so that activity increased. The apparently higher activity of the BR3 isoform detected in radicles isolated from seeds imbibed for 4 h may be an artifact resulting from low seed water content very early in imbibition time and therefore a higher relative protein content.

Although multiple chitinase isoforms were detected both in developing seeds and imbibed seeds, the primary sequences of these isoforms were not investigated in this study; hence, the genes responsible for these enzymes are not yet known. Whether the AD1 acidic chitinase isoform detected in developing and in mature seeds are the same as the major isoform AE1 detected in the endosperm tissue is not yet known. However, the AE1 isoform present in the endosperm might be the AD1 isoform present in the developing seeds, as the AE1 was also present in endosperm tissue isolated from seeds imbibed for only 2 h; and AD1 was present in the fresh mature seeds before imbibition. It is also quite possible that BD1 and BD2 basic isoforms present in developing or mature non-imbibed seeds are the same as two isoforms BE1 and BE2 present in the embryo for the same reason. Since the embryo consists of both embryonic axis and cotyledons, BE1 and BE2 isoforms detected in the embryo tissue are likely to be the same as the two isoforms BR1 and BR2 detected in the embryonic axis prior to germination. Further work will be needed to determine the identity of these multiple isoforms.

Chitinase has been found in a few seeds during imbibition under normal germination conditions; and, in some of them, chitinase is regulated tissue specifically. In pea seeds, high constitutive levels of chitinase activity were present in embryonic axis and cotyledon tissue during seed germination (Petruzzelli et al., 1999). The highest chitinase activity was detected in the embryonic axis in imbibed seeds. In barley seeds, at least five chitinase isoforms were also present. Two chitinases (CH1 and CH2) were more abundant in the aleurone and endosperm tissues, while the other three (CH3, CH4,

and CH5) were predominantly in the embryo tissue, indicating that they are regulated tissue-specifically (Swegle et al., 1992). In carrot seeds, chitinase was present uniformly in the endosperm during imbibition (van Hengel et al., 1998). Chitinase has also been shown to be differentially expressed and induced in combination with  $\beta$ -1, 3 glucanase in response to fungal infection during the germination of maize seeds (Cordero et al., 1993). Three basic chitinase isoforms were induced in germinating wheat seeds infected with *Fusarium culmorum* (Caruso et al., 1999). In all these studies, chitinase has been suggested to play a role in seed defense. Our study gave additional support to this suggestion. It is reasonable to predict that seeds would have protective mechanisms to improve their chances of developing into healthy seedlings, especially when grown in environments inhabited by pathogens. Producing chitinases may be one of the defense mechanisms seeds have.

We detected chitinase activity in dry non-imbibed muskmelon seeds. Chitinase in dry seeds may prevent insect predation, or it may also be stored to protect seeds when they begin to germinate. Insect guts are composed of chitin in many cases, and chitinases may disrupt or interfere with insect feeding. Preliminary research from the University of Arizona showed that white flies fed muskmelon seeds. However, stored grains are susceptible to insect attack even though they do contain substantial levels of chitinases (Molano et al., 1977; Leah et al., 1987), suggesting that insects may have adapted to overcome the effects of plant chitinases (Kramer et al., 1997). Chitinase itself may not be sufficient in defending seeds against insect predation (as will be discussed fully later), or

other defense mechanisms may exist. Recent studies have shown that storage proteins such as vicilins present in legume seeds like some cowpea, adzuki bean, jack bean and soybean have the ability to bind strongly to several chitin-containing structures including the midgut of both *C. maculatus*, *Z. subfasciatus* and chitin (Firmino et al., 1996, Yunes et al., 1998). Vicilins from these distantly related species exhibited a highly detrimental effect on larval developmental of *C. maculatus* (Sales et al., 2000).

In most seeds, endosperm tissue provides storage reserves for the developing seedling. In muskmelon seeds, the endosperm has apparently evolved to also play a protective role by shielding the vulnerable embryo from the harsh chemical environment in the decaying fruit (Welbaum and Bradford, 1990). The endosperm has semi-permeable characteristics that prevent electrolyte leakage from the embryo into the surrounding environment. This characteristic helps deter fungal pathogens that are attracted to sugars that diffuse from the imbibing embryo during germination. The endosperm tissue could also act as a physical barrier to protect the embryo from pathogenic attack, because it has thick cell walls that are suberized and covered with a layer of callose (Yim and Bradford, 1998). Biochemical compounds, such as chitinases deposited in endosperm tissue, may also function to protect young embryos from pathogen attack. Even if pathogens break the first line of defense provided by the endosperm, the young embryo still could protect itself by releasing basic chitinases that would degrade cell walls of fungal pathogens. In fact, it was found that two embryo-associated chitinases from barley seeds are selectively released into the surrounding

medium during early stages of imbibition (Swegle et al., 1992). In nature, these enzymes would be released into the soil where they could contact soil-borne fungi, possibly causing direct inhibition of mycelial growth. The embryonic axis is the tissue that develops into the radicle as seeds germinate. The radicle is the first tissue that penetrates the endosperm when seeds germinate, so it would be beneficial for the embryonic axis or radicle to have its own isoforms of chitinase to fend off pathogens in the soil during the first critical stages of seedling establishment.

A fungal inhibition assay was performed to test whether chitinase present in crude protein extracts could inhibit the growth of certain fungi. The crude protein extracts from imbibed seeds were added to paper discs placed on agar plates containing *Pythium ultimum*, *Rhizoctonia solani*, *Phytophthora infestans*, and *Trichoderma viride*. However, no inhibition of fungal growth was observed in these experiments (data not shown). This suggests that chitinase activity present in the crude protein extracts is not sufficient to have any effect on the growth of fungi listed above, and purified enzyme may be needed. Studies of chitinases from pea tissue showed that protein extracts containing low chitinase activity did not inhibit the growth of *Trichoderma viride*, while purified chitinase did (Mauch et al., 1988). The antifungal inhibition assay may also indicate that chitinase alone is not sufficient to inhibit the growth of fungi. Studies have shown that inhibition of most fungi required the presence of a combination of chitinases and  $\beta$ -1, 3 glucanases (Mauch et al., 1988). However, we were unable to detect the presence of any  $\beta$ -1, 3 glucanase activity in imbibed muskmelon seeds via gel diffusion assay. However,

$\beta$ -1, 3 glucanase activity in tomato seeds was easily detected by this assay (data not shown).

Both class II and class III chitinases exist in germinated muskmelon seeds

The predicted amino acid sequence of *Cmchi1* cDNA shows very high identity to class III chitinases from *Cucumis sativus*, *Vigna angularis*, and *Hevea brasillensis* (Fig. 3-12), indicating that the *Cmchi1* cDNA encodes a class III chitinase. When compared to class I chitinase, the deduced protein encoded by *Cmchi2* lacks an N-terminus cysteine-rich domain that is considered the chitin-binding domain in other studies (Shinshi et al., 1990), indicating that *Cmchi2* cDNA encodes a class II chitinase.

The presence of a short carboxy-terminal extension of about six amino acids has been shown necessary for vacuolar targeting (Neuhaus et al., 1991b). Neither *Cmchi1* or *Cmchi2* protein possesses a carboxy terminal extension; but each possesses a signal peptide, suggesting that they might be targeted for secretion.

Sequence comparison between the *Cmchi2* cDNA clone and the *Cmchi2* partial genomic clone indicates that there are two introns present in the coding region of the *Cmchi2* gene. Although most chitinase genes do not contain introns, some do possess introns. In *Arabidopsis*, one acidic chitinase gene had two introns, while a basic chitinase gene had one intron (Samac et al., 1990). Compared to the *Cmchi2* gene, *Cmchi1* appears to be intronless. This is because the PCR product amplified from the genomic template is the same size as the PCR product amplified from the cDNA template (Fig. 3-

6). One might suspect that contamination could be present so that both PCR products could be amplified from the same cDNA template. However, when water was used as template for PCR in combination with the other two PCR reactions, no product was obtained, indicating that the primers or other reagents were not contaminated. *Cmchi1* cDNA showed 92% identity at the nucleotide level to a cDNA encoding a class III chitinase cloned from cucumber (Metraux et al., 1989). Using this cDNA to screen the cucumber genomic library, three intronless genes encoding class III chitinases were found in the cucumber genome (Lawton et al., 1994). We conclude that *Cmchi1* gene is at least intronless within its coding region.

#### The gene or gene family encoding chitinases existing in muskmelon seeds

Genomic DNA filter hybridization analysis (Southern Blotting) was carried out to establish the copy number of both *Cmchi1* and *Cmchi2* chitinase genes and also to test for the presence of other related chitinases. The three restriction enzymes (BamH I, EcoR I and Hind III) used to determine the number of copies of *Cmchi1* existing in the muskmelon genome were selected for two reasons (the situation for *Cmchi2* is complex and will be discussed fully later). First, recognition sites for these three restriction enzymes were absent from cDNA according to the sequence analysis. Therefore, these enzymes lack recognition sites in the probed regions of the genomic DNA for *Cmchi1*, as this gene is intronless. Second, based upon preliminary results, BamH I, EcoR I, and Hind III completely digested muskmelon genomic DNA. Some restriction enzymes do not completely digest muskmelon genomic DNA as different restriction enzymes might

have different sensitivities to site-specified methylation of DNA, and methylation of DNA is more extensive in higher plants than in animals (Zhu et al., 1994).

Southern blot analysis was carried out under low-stringency conditions. Two strongly hybridizing bands were seen for BamH I, EcoR I or Hind III digestion when the membrane was probed with DIG-labeled *Cmchi1* cDNA (Fig. 3-15). This indicates that there is at least another gene similar to *Cmchi1* existing in the muskmelon seed genome, as *Cmchi1* gene is intronless and should not be cleaved internally to produce two or more bands. This is consistent with an earlier report that there are three very closely related genes encoding class III chitinases in cucumber (Lawton et al., 1993). The function of multiple isoforms may be to allow seeds to respond to environmental changes in a tissue or stimulus-specific manner. For example, chitinase Ch1 in pea is expressed during fungal attack and after ethylene treatment, while a different chitinase, Ch2 is expressed primarily in maturing seed pods (Mauch et al., 1988).

Southern blot analysis revealed that one strongly hybridizing signal was detected when genomic DNA was digested with BamH I, EcoR I, and Hind III and probed with DIG-labeled *Cmchi2* cDNA (Fig. 3-16). We believe there is only one copy of the *Cmchi2* gene in the muskmelon seed genome for the following reasons. *Cmchi2* is 1394 bp in length with two introns in its coding region. According to sequence analysis, EcoR I has two restriction sites in the genomic clone, one starting at nucleotide 78 and the other one starting at nucleotide 1351 in terms of its position in the partial genomic clone

sequence. Digestion of genomic DNA with EcoR I would give rise to three fragments, i. e., if there is one copy of the gene existing in the genome, a fragment containing 78 bp of the genomic sequence of *Cmchi2*, a fragment with 1273 bp, and another fragment containing 53 bp of genomic sequence of *Cmchi2*. When this digest was probed with DIG-labeled *Cmchi2* cDNA, only one hybridization signal was detected. Because the two restriction sites were beyond the probing region (probe starts at nucleotide 94 and ends at 1346 in terms of its position in the partial genomic clone sequence), these two fragments would not hybridize with DIG-labeled *Cmchi2* cDNA; only the 1273 bp fragment would hybridize to the probe. There was only one hybridization signal, indicating that there is only copy of *Cmchi2* existing in the genome.

Digestion of genomic DNA with Hind III and probed with DIG-labeled *Cmchi2* cDNA also generated one strong hybridization signal. Hind III has two restriction sites within the genomic DNA clone sequence, with one site starting at nucleotide 121 and another site starting at nucleotide 293. Digestion of genomic DNA with Hind III would also give rise to three fragments, if there is one copy of the gene in the genome, that is, a fragment containing 121 bp of genomic sequence of *Cmchi2*, a fragment with 172 bp, and a large fragment containing 1101 bp of the genomic sequence of *Cmchi2*. During electrophoresis, the 172 bp fragment could run off the gel and therefore would not be able to hybridize to the probe. The fragment containing 121 bp of the genomic sequence of *Cmchi2* only contains 27 bp that could be hybridized to the probe. This hybridization

might not be strong enough to give a strong signal. This is further evidence to support that there is only one copy of *Cmchi2* in the genome.

According to sequence analysis, there is a BamH I site in the genomic clone. When the cDNA sequence was compared with the genomic clone, there was a mismatch between these two sequences. A BamH I site occurred in the exon region of the genomic clone but not in the cDNA because of the mismatch. Whether there is a BamH I site is not known at this point, however, digestion of genomic DNA with BamH I gave rise to a hybridization signal which might indicate that there is no BamH I site in the *Cmchi2* gene. This is additional evidence to support the fact that only one copy of *Cmchi2* exists in the muskmelon genome.

The presence of a single copy of *Cmchi2* in the muskmelon genome indicates that regulation of the promoter of this gene may be very complex. Deletion analysis of the promoter region will help identify *cis*-acting elements in the promoter region that are responsive to different tissue-specific and environmental signals.

$\beta$ -Actin can be used as a loading control for study of gene expression.

Since we could not detect the expression of both *Cmchi1* and *Cmchi2* using total RNA under our experimental conditions, poly (A<sup>+</sup>) RNA was used instead. A similar problem also occurred in studies of maize seeds in which the level of chitinase clone CHTA was only detected by using up to 5  $\mu$ g poly (A<sup>+</sup>) RNA (Huynh et al., 1992). A  $\beta$ -Actin7 probe made from the middle region of *Arabidopsis thaliana* ACT7 gene was used

for the loading control. Sequence alignment of the  $\beta$ -Actin7 gene from *Arabidopsis* and actin genes from other species indicated that this gene is highly conserved among different species. Although  $\beta$ -Actin7 gene has not been cloned from muskmelon seeds, it is likely that this gene is a housekeeping gene and would be expressed in muskmelon seed. Therefore *Arabidopsis*  $\beta$ -actin 7 was used as a loading control in this study with the expectation that this heterogenous probe would hybridize with the actin gene expressed in muskmelon.

The expression of both *Cmchi1* and *Cmchi2* is coupled with radicle emergence and regulated in a tissue-specific manner.

The expression of both *Cmchi1* and *Cmchi2* was detected only at the time when the radicle emerges. In fact, both genes were expressed in the radicle and not in the remaining seed tissue (Fig. 4-2). The expression of both *Cmchi1* and *Cmchi2* was not detected in seeds at very early imbibition times and was also not detected in developing seeds. Since radicles develop into roots, it is likely these genes are expressed in roots as well. Interestingly, *Cmchi1* and *Cmchi2* were present in roots only but not in other tissues such as leaves, stems, and flowers (Fig. 4-3). We believe that these two genes are coupled to radicle emergence, and that their expression is restricted to radicle and root tissues. During muskmelon seeds germination, disruption of endosperm tissue is necessary in order to allow the radicle to emerge from seeds. This physical disruption of tissue may lead to chitinase gene expression.

Chitinases have been found in roots of some other higher plants. In tobacco roots, chitinases constitute as much as 4% of the total soluble protein (Shinshi et al., 1987). In the roots of 11-day-old cucumber seedlings, six acidic chitinases were present and two were unique to roots (Majeau et al., 1990). However, so far no study has shown that chitinase is expressed only at the time of radicle emergence soon after germination. The presence of chitinase in radicle tissue may slow the growth of soil borne pathogens attacking plants in the field as the radicle is the first tissue to emerge from a seed and contact the soil. The induction of chitinase gene expression in radicle tissue after germination indicates that seeds may have the ability to sense the change of germination phase, and activate their defense mechanisms to prevent potential pathogenic attack.

Neither cell-types nor subcellular localization of *Cmchi1* and *Cmchi2* gene expression in radicle tissue was determined in this study. In pea, in situ hybridization has shown that a pathogenesis-related gene is expressed in root hair epidermis cells (Mylona et al., 1994), suggesting a constitutive defense barrier in the root epidermis. If these two genes were expressed in the root hair, which is in intimate contact with the soil, it would provide even stronger evidence to support the defense mechanism. In situ hybridization, specific expression of a reporter gene driven by promoters of these genes and immunolocalization will help to reveal the cell type or subcellular localization of these gene products.

SA and BTH stimulated the expression of *Cmch1* but not *Cmchi2* in imbibed muskmelon seeds and in seeds after radicle emergence.

Chitinase has been used as a molecular and biochemical marker for the induction of local and systemic acquired resistance (SAR) in higher plants. SAR is the protective response acquired by plants to protect against subsequent infection by unrelated pathogens when infected with necrotic pathogens. Chitinase is correlated with SAR in a number of plant species such as cucumber and tobacco (Métraux et al., 1989; Lawton et al., 1994; Shinshi et al., 1990). SAR can be induced in higher plants by compounds such as SA or its analog BTH (Uknes et al., 1992; Görlach et al., 1996). Chitinase present in cucumber leaves can be greatly stimulated after treatment with SA (Lawton et al., 1994). In wheat, BTH was found to be more effective at inducing gene expression and disease resistance (Görlach et al., 1996).

Most of the studies on SAR have been conducted in mature higher plants, research on the induction of gene expression by SA or its analog BTH in germinating seeds or very young seedlings is very limited. Induction or stimulation of chitinase production in germinating seeds or very young seedlings would have profound importance for US agriculture where expensive chemicals are applied annually as seed treatments to protect seedlings from soil-borne pathogens.

SA or BTH could stimulate the level of *Cmchi1* mRNA in muskmelon seeds during imbibition, especially soon after radicle emergence. As shown in Figure 4-4, the

level of *Cmchi1* mRNA increased slightly when seeds were imbibed with 1 mM SA for 16 h and increased dramatically when seeds were imbibed for 24 h. When muskmelon seeds were imbibed with 0.3 mM BTH, the level of *Cmchi1* mRNA increased in seeds imbibed for 16 and 24 h but not 8 h. As stated before, *Cmchi1* shows very high identity to a class III chitinase gene from cucumber. In cucumber, the expression of this class III gene can be induced by application of SA (Lawton et al., 1994). The application of SA resulted in a 10-fold increase in class III chitinase mRNA within 1 day. Our results are consistent with the result for the class III chitinase gene in cucumber. This result suggests that SA might be involved in the signal transduction pathway leading to the expression of *Cmchi1* gene.

Imbibed seeds treated with 1 mM SA or 0.3 mM BTH did not increase the level of *Cmchi2* mRNA at any time (Fig. 4-5). *Cmchi2* encodes a basic class II chitinase. It was found in several studies that only acidic chitinases but not basic chitinases were preferentially induced by SA treatment (Buchter et al., 1997; Ancillo et al., 1999; Brederode et al., 1991). In potato, the expression of an acidic class II chitinase gene, ChtA, was preferentially induced by application of SA, while the expression of a basic class I chitinase gene, Chtb, was induced by ethylene or wounding (Buchter et al., 1997). The expression of another chitinase gene, ChtC, encoding a basic class I chitinase from potato was not induced by SA treatment but was induced by other stimuli such as fungal elicitors, ethylene or wounding (Ancillo et al., 1999). In tobacco, SA also induced the highest expression of acidic chitinases but not basic chitinases (Brederode et al., 1991).

Since the predicted protein encoded by *Cmchi2* is a basic chitinase, the expression of *Cmchi2* may not be regulated by a chemical compound such as SA or BTH but may be regulated by other stimuli such as wounding or ethylene. This indicates that a different signal transduction pathway might be involved in leading to the expression of the *Cmchi2* gene in muskmelon seeds.

The level of B-actin shown in Figure 4-5 and 4-6 increased slightly with increasing imbibition time. This apparent increase may be due to experimental error. Alternatively, either SA or BTH may affect the expression of  $\beta$ -actin, however, this has not been reported.

#### Expression of *Cmchi1* in *E. coli*

To test whether the predicted protein identity of *Cmchi1* is correct, *Cmchi1* cDNA was expressed in *E. coli*. The protein was initially expressed in inclusion bodies and was subsequently found in the insoluble fraction after sonication (Fig. 4-6). The protein was renatured and purified from an amylose resin column (Fig. 4-6). When gel diffusion assay was performed using both the purified protein and the cleaved fusion protein, no enzyme activity was detected. A chitinase gene cloned from tomato was expressed using the same expression system and the fusion protein showed enzyme activity (personal communication from Chun-Ta Wu in UC Davis). It is possible that the fusion protein might not have folded properly for catalytic function. It is also possible that post-translation modification might be required for its catalytic function. The inactivity of this

protein prevented the conclusive demonstration that *Cmchi1* encodes a chitinase. It also prevented further experiments with antifungal assays with large quantities of purified proteins.

Western blotting using an antibody raised against the class III cucumber chitinase protein in cucumber showed that this antibody recognized the purified *Cmchi1* fusion protein (not shown). The antibody also detected the presence of other unknown proteins in crude proteins isolated from muskmelon embryonic axis or the radicle tissue as well. The experimental conditions may need to be optimized in order to detect the *Cmchi1* protein in muskmelon seeds.

The connection between the chitinase activity detected and the genes cloned in muskmelon seeds is unknown

Although we have demonstrated that there is chitinase activity in muskmelon seeds and we also have cloned and sequenced genes encoding class II and class III chitinases in seeds after radicle emergence, the connection between the activity detected and the genes cloned still remains to be determined. It is possible that *Cmchi1* might encode an acidic chitinase detected in the endosperm tissue as the acidic isoform is only detected in the endosperm tissue and the *Cmchi1* encodes an acidic chitinase because of its predicted low pI of 4.3. Purification, peptide massing or N-terminal sequencing of the acidic protein will allow us to determine whether there is a connection between the acidic chitinase existing in the endosperm tissue and the cloned *Cmchi1*. A similar

approach could also be taken to discover the connection between basic chitinase in the endosperm or embryo tissue and the cloned *Cmchi2*.

## CONCLUSIONS

This dissertation has characterized chitinase activity and gene expression in muskmelon seeds. Our study has shown that chitinase activity exists in developing, mature, and dry muskmelon seeds, and in seeds during imbibition and after radicle emergence. Chitinase has been suggested to be involved in the defense response in higher plants. We believe that chitinases in muskmelon seeds may play a role in seed protection during development, imbibition and after radicle emergence.

Multiple chitinase isoforms were detected in muskmelon seeds. These isoforms are regulated developmentally and in a tissue-specific manner. One acidic and three basic chitinase isoforms were detected in developing seeds at 40 DAA, suggesting a potentially preformed defense mechanism during seed development. Both acidic and basic chitinase isoforms are detected in endosperm tissue, indicating that the endosperm may provide a biochemical as well as a physical defense mechanism to protect the young embryo. This is especially true for the muskmelon seed endosperm because of the following characteristics. First, muskmelon seed endosperm has semi-permeable characteristics that prevent electrolyte leakage from the embryo into the surrounding environment (Welbaum and Bradford, 1990; Yim and Bradford, 1998). These characteristics help deter fungal pathogens that are attracted to sugars that diffuse from the imbibing embryo during germination. Second, the muskmelon endosperm has thick

cell walls that are suberized and covered with a layer of callose (Yim and Bradford, 1998).

Basic chitinase isoforms, but not acidic isoforms, were detected in muskmelon embryos. Within the embryo, activity of basic chitinase isoforms were found in the embryonic axis (pregermination) or the radicle tissue (postgermination), while activity from the cotyledon was not evaluated. The radicle is the first tissue that penetrates the endosperm when seeds germinate and hence the embryonic axis or radicle may protect itself by secreting chitinase into the surrounding soil during the critical stages of seedling establishment.

Two cDNAs and a partial genomic clone have been isolated from muskmelon seeds after radicle emergence. Sequence comparison between the *Cmchi2* cDNA clone and the *Cmchi2* partial genomic clone indicates that there are two introns present in the coding region of the *Cmchi2* gene, while *Cmchi1* is intronless. Southern blot analysis suggests that there is at least one other gene similar to *Cmchi1* in the muskmelon genome, while there is one copy of *Cmchi2*. *Cmchi1* cDNA encodes a class III chitinase, as the predicted amino acid sequence of *Cmchi1* cDNA shows very high identity to class III chitinases from *Cucumis sativus*, *Vigna angularis*, and *Hevea brasillensis*. *Cmchi2* cDNA encodes a class II chitinase, as the predicted amino acid sequence of *Cmchi2* cDNA shows high identity to both class I and class II chitinases but it lacks an N-terminus cysteine-rich domain, which is characteristic of class I chitinases. *Cmchi1* and

*Cmchi2* proteins might be targeted to a secretory pathway, as the amino acid sequences of both *Cmchi1* and *Cmchi2* were predicted to possess signal peptides but not carboxy terminal extensions.

The expression of both *Cmchi1* and *Cmchi2* is closely linked to germination, expressed at the time of radicle emergence and regulated in a tissue-specific manner. Both genes were expressed in the radicle and not in the remaining seed tissue. Interestingly, *Cmchi1* appears to be more highly expressed in roots than *Cmchi2*. Neither gene was expressed in leaves, stems, or flowers. We propose that these two genes are expressed at the time of radicle emergence and they are specifically expressed only in radicle and root tissues. The presence of chitinase in radicle tissue may slow the growth of soil-borne pathogens attacking plants in the field. The induction of chitinase gene expression in radicle tissue after germination indicates that seeds may have the ability to sense the change of germination phase and activate their defense mechanisms to protect against pathogenic attack.

The induction of *Cmchi1* or *Cmchi2* gene expression in imbibed seeds and in seeds soon after radicle emergence was investigated in this study. SA and BTH stimulated the expression of *Cmchi1* but not *Cmchi2* in imbibed muskmelon seeds and in seeds soon after radicle emergence. This suggests that SA might be involved in signal transduction leading to the expression of *Cmchi1* gene, while a different signal transduction pathway might be involved in the expression of *Cmchi2* gene in muskmelon

seeds. Strategies aimed at stimulating chitinase production in germinating seeds or very young seedlings would be profoundly important to agriculture. If natural defense mechanisms in seeds could be stimulated and resistance increased, the amount of chemical seed treatments applied to seeds could be reduced.

Although *Cmchi1* is predicted to encode a chitinase, further experimental evidence was sought to confirm the identity of the predicted *Cmchi1* protein. The *Cmchi1* protein expressed in *E. coli* did not show any enzymatic activity. It is possible that the fusion protein may not have folded properly for catalytic function. It is also possible that post-translation modifications are necessary for catalytic function. The lack of enzyme activity prevented a direct identification of the *Cmchi1* gene product as chitinase and also antifungal testing with purified protein.

Western blotting using an antibody raised against the class III chitinase protein in cucumber showed that this antibody recognized the purified *Cmchi1* fusion protein. The antibody also detected other unknown proteins in crude extracts isolated from the embryonic axis or the radicle tissue as well. The experimental conditions may need to be optimized in order to detect the localization of the *Cmchi1* protein in muskmelon seeds.

Although we have demonstrated the existence of chitinase activity in muskmelon seeds and we have also cloned genes encoding class II and class III chitinases expressed in the radicle and roots, the connection between the activity detected and the genes cloned still remains to be determined. It is possible that *Cmchi1* encodes an acidic

chitinase detected in the endosperm tissue, as the acidic isoform is only detected in the endosperm tissue and the *Cmchi1* is predicted to encode an acidic chitinase. Purification and sequencing of the acidic protein would allow us to determine the connection between the acidic protein found in the endosperm tissue and the cloned *Cmchi1*. A similar approach could be taken to discover the connection between basic chitinase in the endosperm or embryo tissue and the cloned *Cmchi2*.

In summary, multiple isoforms were present in the muskmelon seeds during development, imbibition, and after radicle emergence. These isoforms were regulated developmentally and in a tissue specific manner. Two chitinase cDNAs, *Cmchi1* and *Cmchi2* and a partial genomic *Cmchi2* gene, have been cloned from muskmelon seeds. *Cmchi1* and *Cmchi2* are expressed in seeds after radicle emergence. The expression was restricted to the radicle and root tissue. SA and BTH stimulated the expression of *Cmchi1* but not *Cmchi2* in seeds after radicle emergence.

Table 5. Summary of chitinase activity and different isoforms present in developing seeds, mature seeds, seeds during imbibition and after radicle emergence.

Whole seeds or seed tissue	Developing seeds (DAA)				Mature seeds		Seeds during incubation (h)														
	20	30	40	50	Fresh	Dry	before radicle emerges					after radicle emerges									
							2	4	8	12	16	20	24	28	32	48					
Whole seeds	-	-	1A 3B	1A 3B	1A 3B	+															
Endosperm							1A 2B	1A 2B	1A 2B	1A 2B	2A 2B	2A 2B	2A 2B	2A 2B	2A 2B	2A 2B	2A 2B	2A 2B	2A 2B	2A 2B	
Embryo							-A 3B	-A 3B	-A 3B	-A 3B	-A 3B	-A 3B	-A 3B	-A 3B	-A 3B	-A 3B	-A 3B	-A 3B	-A 3B	1A 3B	
Embryonic axis								3B			2B				2B						
Radicle																				1B	1B

-, Activity not detected; +, Activity detected; 1A, 2A, Numbers of acidic chitinase isoform; -A, No acidic isoform detected; 2B, 3B, Numbers of basic chitinase isoform; Empty boxes, Activity not assessed.

Table 6. Summary of gene expression in developing muskmelon seeds, seeds during incubation with water, SA, or BTH at different times and in different tissues of mature plants.

Gene	Deve- loping seeds (DAA)		Seeds during incubation (h)										Mature plant tissues							
			Water					SA treatment		BTH treatment			Roots	Leaves	Stems	Flowers				
	25	35	8	16	20	24	48	8	16	24	8	16	24	-	+	-	+	-	-	-
<i>Cmchi1</i>	-	-	-	-	+	+	+	-	+	+	-	+	+	+	+	-	+	-	-	-
<i>Cmchi2</i>	-	-	-	+	+	+	-	+	+	+	-	-	+	+	+	-	+	+	-	-

-, No expression detected; +, expression detected; ND, Expression not determined; \* , Expression increased after treatment

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