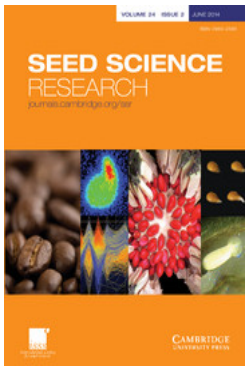

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Characterization of chitinase activity and gene expression in muskmelon seeds

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Abstract

Chitinase is often produced in higher plants as a general defence response after wounding or pathogenic attack. Since germinating seeds are exposed to soil pathogens, the activity and expression of chitinase in muskmelon (*Cucumis melo* L.) seeds was investigated. One acidic and three basic chitinase isoforms were detected, beginning 40 d after anthesis in developing and fully mature seeds. Both acidic and basic chitinase isoforms were found in endosperm tissue during imbibition and after radicle emergence. Basic chitinase isoforms, but not acidic isoforms, were detected in the embryonic axes of imbibed seeds and in seeds before germination, indicating that chitinases are developmentally regulated in specific seed tissues. Two complete cDNAs, *Cmchi1* and *Cmchi2*, were cloned from germinated muskmelon seeds and are predicted to encode chitinases that show 95% identity to a class III chitinase from cucumber (*Cucumis sativus* L.) and 61% identity to a class II chitinase from soybean (*Glycine max* L.), respectively. Southern blotting indicated that *Cmchi2* was present only once in the muskmelon genome, while *Cmchi1* may be present in one or two copies. *Cmchi1* and *Cmchi2* mRNAs were only detected in radicles of germinating seeds and in roots of mature plants, so additional genes other than *Cmchi1* and *Cmchi2* must be responsible for the chitinase activity in developing seeds. Salicylic acid and benzothiadiazole stimulated the expression of *Cmchi1*, but not *Cmchi2*, after radicle emergence. A putative role for chitinase in muskmelon seeds is defence against fungal pathogens.

Keywords: *Cucumis melo*, chitinase, defence response, seed germination, systemic acquired resistance

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Introduction

Natural defence mechanisms protect seeds from abundant soil-borne pathogenic microorganisms that colonize many soils. In some seeds, specialized tissues provide a physical barrier to protect against pathogenic attack. In muskmelon (*Cucumis melo* L.) seeds, for example, nutrient leakage from the embryo during imbibition, which could stimulate microbial growth around the seed, is reduced by callose deposits that create a semi-permeable endosperm cell wall (Welbaum and Bradford, 1990; Yim and Bradford, 1998). In addition, the thick cell walls of the endosperm tissue may also provide a physical barrier to slow penetration of fungal hyphae (Welbaum and Bradford, 1990; Yim and Bradford, 1998). However, the embryo becomes vulnerable to pathogenic attack when the expanding radicle breaks through the endosperm tissue during germination. It is, therefore, likely that a post-germination defence strategy protects germinating seeds once physical barriers are compromised (Fincher, 1989; Flach *et al*., 1992).

Chitinases accumulate in seeds of several species as part of their developmental programme, while others can be induced in response to microbial attack (reviewed by Gomez *et al*., 2002). Seed chitinases may protect against chitin-containing pathogenic fungi, because substrates for chitinase are found in some fungal cell walls, but not in plants (Powning and Irzykiewicz, 1965; Graham and Sticklen, 1994; Gomez *et al*., 2002). However, the finding that a chitinase could rescue a somatic embryo mutant is evidence of a non-defence function as well (De Jong *et al*., 1992, 1993). In soybean (*Glycine max* L.), a class I chitinase was expressed in the seed coat, pod, root, leaf and embryo late in development (Gijzen *et al*., 2001), and chitinases have been detected in dry seeds (Ramos *et al*., 1998; Krishnaveni *et al*., 1999). Barley (*Hordeum vulgare* L.) grains released chitinases into the surrounding medium during the early stages of imbibition (Swegle *et al*., 1992). Chitinase gene expression is induced in response to fungal infection

during germination of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) caryopses (Cordero *et al.*, 1994; Caruso *et al.*, 1999). In tomato (*Lycopersicon esculentum* L.) seeds, a class I basic chitinase was expressed specifically in the micropylar endosperm just prior to radicle protrusion (Wu *et al.*, 2001).

In this study, we identified various chitinase isozymes present in developing and germinating muskmelon seeds. We also characterized two cDNAs, *Cmchi1* and *Cmchi2*, which encode class III and class II chitinases, respectively, in the radicles of germinating muskmelon seeds. Seeds were also treated with salicylic acid or benzothiadiazole to assess whether *Cmchi1* or *Cmchi2* expression was affected by compounds that induce systemic acquired resistance (Lawton *et al.*, 1995; Görlach *et al.*, 1996).

Materials and methods

Plant material and seed germination

Muskmelon (*Cucumis melo* cv. Top Mark) seeds were decoated by hand and germinated at 30°C in the dark on 100 × 15 mm Petri dishes with three layers of germination blotter paper (Anchor Paper Co.) moistened with 20 ml distilled water, 0.3 mM benzothiadiazole (BTH), or 1 mM salicylic acid (SA) for 2–48 h. Some decoated imbibed seeds were dissected to separate the endosperm envelope from embryonic tissues. In other cases, decoated germinating seeds were dissected to separate the radicle from the remaining seed tissues.

Protein extraction

Proteins were extracted from seeds (5 g) during imbibition and after germination or from isolated seed tissues (5 g) 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 10,000 *g* for 5 min at 4°C, and the supernatant was assayed immediately for enzyme activity via gel diffusion using glycol chitin as the substrate (Zou *et al.*, 2002).

Native-PAGE and activity staining for chitinases

Native polyacrylamide gel electrophoresis (native-PAGE) was performed in a 7.5% (w/v) polyacrylamide gel containing 0.5% glycol chitin as the substrate. Protein extracts (20 µl) were separated by electrophoresis at either pH 8.8 for acidic chitinases or pH 4.3 for basic chitinases. After electrophoresis, the gels were equilibrated in 0.1 M 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 (Trudel and Asselin, 1989).

Degenerate primers for amplifying chitinase cDNAs

Degenerate primers were designed from conserved domains within chitinases from several different plant species. To identify conserved domains for class I and II chitinases, chitinase protein sequences from different species were retrieved from the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/). Amino acid sequence alignment was performed with accession numbers AB018248, AB012855, X87109, 20202, L34211, 19191, 19845, A16119, A21091, 1729760, Z54234, Y10373, Z55452, Z70032, and 388509 (multiple sequence alignment program from LASERGENE). 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 and used for degenerate primer design. The degenerate primers for these 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). Another amino acid sequence alignment was performed for class III chitinases with accession numbers AJ007701, AJ010397, D55713, D49953, 17942, S31763, 99621, and D55711. Degenerate upstream (5′-GGC RTB TAY TGG GGN CAR AA-3′) and downstream (5′-GGR TTR TTR TAR AAY TGN ACC ACA-3′) primers were designed from two conserved regions (A I/V Y W G Q N and W V Q F Y N N).

Cloning of cDNAs using RT-PCR, 3′ RACE and 5′ RACE

Using total RNA isolated from developing seeds at 10, 20 and 30 d after anthesis (DAA) or mature seeds imbibed for 16, 24, 32 or 48 h as templates, reverse transcriptase (RT) reactions were performed (RETROscript™, Ambion, Austin, Texas, USA). Polymerase chain reaction (PCR) was performed using the degenerate primers described above: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min (32 cycles) (Robocycle Gradient Thermocycler, Stratagene, La Jolla, California, USA). The PCR products with expected sizes were cloned (pGME®-T Easy Vector, Promega, Madison, Wisconsin, USA) according to the manufacturer's recommendations. The 3′ and 5′ ends of the RT-PCR products (*Cmchi1* and *Cmchi2*) were amplified using 3′ and 5′ RACE (Rapid Amplification of cDNA Ends, Life Technologies, Rockville, Maryland, USA). Gene-

specific primers for amplifying the 3′ end of *Cmchi1* were 48AP (5′-AAG GCT CTC TTG CAT CCA CC-3′) and 48EM (5′-GTC AAA GTC CTC CTC TCT ATC G-3′). Gene-specific primers for amplifying the 5′ end of *Cmchi1* were 48GSP1 (5′-TTC AAA ACA GCA GCA CCG A-3′), 48GSP2 (5′-CGA TAG AGA GGA GGA CTT TG-3′), and 48GSP3 (5′-GAC GAA CTC GTA GTT TCC AG-3′). Gene-specific primers for amplifying the 3′ end of *Cmchi2* were 24AP (5′-GCT AAC TGC ACC AGA TGG TC-3′) and 24DM (5′-ATG GTC GTG GAC CAA TGC AA-3′). Gene-specific primers for amplifying the 5′ end of *Cmchi2* were 24GSP1 (5′-CTA AAT CTG GGT TCT TCA GC-3′), 24GSP2 (5′-TTG CAT TGG TCC ACG ACC AT-3′), and 24 GSP3 (5′-TGG TGC AGT TAG CCA TCC TC-3′).

To obtain full-length cDNAs, primers were designed from sequences generated from 3′ and 5′ RACE for both *Cmchi1* and *Cmchi2*. 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 (close to the 5′ end in the untranslated region) (5′-CAC AAA CTC CAA CCA AAG CTC-3′), and a downstream primer 48RV (close to the 3′ end in the untranslated region) (5′-AGC TTA ATT CAG CCG ATG CTG –3′). PCR conditions were as above. The full-length cDNA for *Cmchi2* cDNA was amplified by PCR using an upstream primer 24FW1 (close the 5′ end in the untranslated region) (5′-CGA ATT TAA AAC TCA GAG AGA AAA-3′), a downstream primer 24RV (close to the 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. In each case, the resulting 1 kb amplified fragment was then cloned (pGME®-T Easy Vector, Promega) and sequenced.

DNA isolation and Southern hybridization

Genomic DNA (20 µg) isolated from leaves of muskmelon seedlings (Dellaporta *et al*., 1983) was digested at 37°C for 3 h with restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII (New England Biolabs, Beverly, Massachusetts, USA) and separated electrophoretically on agarose gels (1%) for 6 h at 70 V. Gels were 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. Gels were soaked in transfer buffer [20× SSC: 3 M NaC1 (125 g l^{-1}), 0.3 M trisodium citrate $2H_2O$ (88 g l^{-1}) adjusted to pH 7.0 with 1 M HCl] for 30 min and transferred to nylon membranes using a rapid downward transfer system (TurboBlotter, Schleicher & Schuell, Keene, New Hampshire, USA). Membranes were cross-linked using the optimal UV setting (UV Crosslinker, Fisher Scientific, Pittsburgh, Pennsylvania, USA), prehybridized with a commercial hybridization buffer (Ultrahyb, Ambion) at 42°C for 1 h, and hybridized at 42°C overnight with digoxigenin (DIG)-labelled *Cmchi1* or *Cmchi2* cDNA probes (PCR DIG Probe Synthesis Kit, Roche Molecular Biochemicals, Indianapolis, Indiana, USA). Following hybridization, membranes were washed twice for 15 min at low stringency (2× SSC, 0.1% SDS, 25°C) and twice at high stringency (0.1× SSC, 0.1% SDS, 42°C). The hybridization signal developed by chemiluminesence using CDP-star substrate (Roche Molecular Biochemicals) was detected on X-ray film (Biomax MR, Kodak, Amersham Biosciences, Piscataway, New Jersey, USA) after a 2–10 min exposure (Chemiluminescent Detection System, Roche Molecular Biochemicals).

RNA extraction and Northern blotting

Total RNA was extracted from imbibed seeds or from roots, stems, leaves and flowers of mature field-grown muskmelon plants, using a standard phenol–SDS method (Sambrook *et al*., 1989), and poly(A^+) RNA was isolated from total RNA (Oligotex mRNA Spin-Columns, QIAGEN, Valencia, California, USA). For RNA probe synthesis, a 598 bp fragment from *Cmchi1* cDNA was amplified using primers 48EM and 48RV. A 527 bp fragment was amplified from *Cmchi2* cDNA using 24DM and 24RV. A T7 promoter was ligated to each of these two fragments (Lign's Kit, Ambion), which served as templates for PCR amplification using the T7 adapter primer 1 (Lign's Kit, Ambion) and a gene-specific primer (48EM from *Cmchi1* and 24DM from *Cmchi2*). DIG-labelled RNA probes were synthesized using the PCR products prepared above as templates (DIG RNA Labeling Kit, Roche Molecular Biochemicals). Poly(A^+) RNA (1.75–5 µg) was separated by electrophoresis on a 1% agarose gel containing 1× denaturing gel buffer (supplied in the NorthernMax Kit, Ambion) for 2 h at 70 V. The RNA was transferred and cross-linked to a positively charged membrane (Hybond-N^+, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) as for Southern blotting. The RNA-blotted membrane was then prehybridized with 10 ml commercial hybridization buffer (Ultrahyb, Ambion) for 1 h and hybridized with DIG-labelled RNA probe (0.1 nM) overnight at 68°C. The membrane was washed at 25°C twice at low stringency for 5 min and twice at 68°C at high stringency for 20 min each (NorthernMax kit, Ambion). The hybridization signal was detected using chemiluminescence as described for Southern blotting. After detection, the same membrane was stripped and reprobed again with a DIG-labelled antisense *β-Actin 7* probe from *Arabidopsis thaliana*.

Results

Multiple chitinase isoforms in muskmelon seeds

An acidic chitinase isoform, termed AD1, appeared in developing whole muskmelon seeds 40 DAA and increased in activity during seed maturation (Fig. 1A). Three basic chitinase isoforms, BD1, BD2 and BD3, were also detected in developing muskmelon seeds beginning at 40 DAA (Fig. 1B). The activity of basic isoform BD1 was greater than that of either BD2 or BD3.

Chitinase activity was also detected in endosperm and embryo tissue of imbibed and germinating seeds. Muskmelon seed germination (radicle emergence) was completed for the first seeds in the population after 16 h at 30°C. One major acidic isoform, AEn1, was detected in the endosperm tissue during imbibition and after radicle emergence (Fig. 2A). Another acidic chitinase isoform, AEn2, was detected in endosperm tissue after 16 h, and its activity increased after radicle emergence. Basic chitinase isoforms, BEn1, BEn2 and BEn3, were also present in the endosperm tissue during seed imbibition and after radicle emergence (Fig. 2B). No acidic chitinase isoforms were detected in embryo tissues from imbibed seeds (Fig. 2C, lanes 2–16); however, one acidic isoform AEm1 was detected in embryo tissue following radicle emergence (Fig. 2C, lanes 32–48). Three basic chitinase isoforms were detected in embryo tissue from imbibed seeds or from seeds after radicle emergence (Fig. 2D). The activities of basic isoforms BEm5 and BEm6 were dominant, while activity of basic isoform BEm4 was barely visible on the gel.

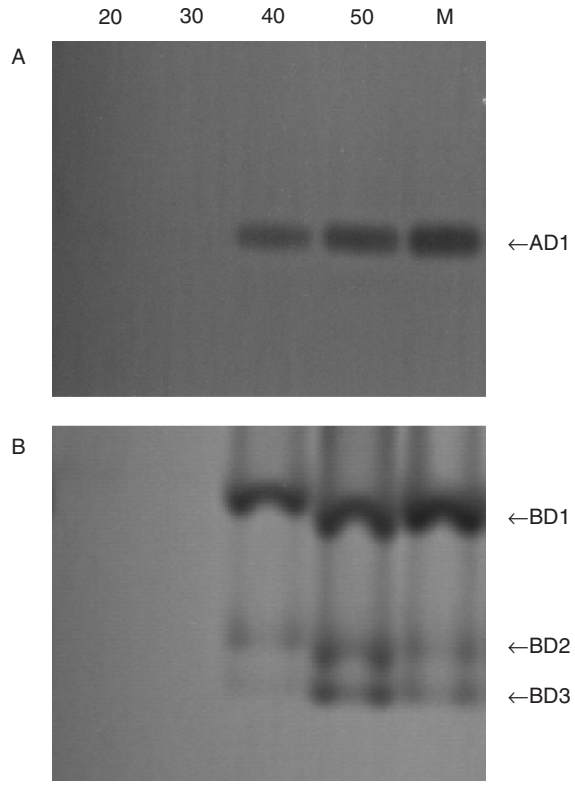

Figure 1. Native-PAGE and activity staining of the acidic (A) and basic (B) chitinase isoforms from non-dried whole muskmelon seeds at different developmental stages. Crude protein extracts were prepared from seeds at 20, 30, 40 and 50 DAA and from fully mature seeds (M). Extracts (20 µl) representing equal fresh weight samples (5 g) were loaded in each lane. Acidic (AD) and basic (BD) isoforms are marked along the gels.

Characteristics of Cmchi1 *and* Cmchi2 *cDNA and predicted proteins*

A cDNA encoding a class III chitinase, *Cmchi1* (GenBank accession No. AF241266), was 991 bp in length, contained a predicted 5′ untranslated region of 39 bp and an ATG initiation codon at position 40, followed by an open reading frame of 879 bp and a 3′ untranslated region of 73 bp. The protein had a predicted molecular mass of 30,784 Da, an isoelectric point of 4.19, a charge of –11.7 at neutral pH, a signal peptide of 25 amino acid residues (Nakai and Kanehisa, 1992), and a catalytic domain of 267 amino acid residues. The deduced amino acid sequence showed 95% identity to a *Cucumis sativus* class III chitinase P17541 (Fig. 3; Métraux *et al*., 1989).

A cDNA encoding the class II chitinase, *Cmchi2* (GenBank accession No. AF241267), was 1038 bp in length, contained a predicted 5′ untranslated region of 24 bp and an ATG initiation codon at position 25, followed by an open reading frame of 819 bp and a 3′ untranslated region of 180 bp. The deduced protein was predicted to have a molecular mass of 30,099 Da, an isoelectric point of 9.28, a charge of 13.3 at neutral pH, a signal peptide of 20 amino acid residues (Nakai and Kanehisa, 1992), and a catalytic domain of 252 amino acid residues. The deduced protein sequence of *Cmchi2* showed 61% identity to the *Glycine max* class I chitinase AF202731 (Fig. 4). However, since *Cmchi2* lacked the chitinase binding domain characteristic of class I chitinases, it is likely a class II chitinase (Gomez *et al*., 2002).

Southern analysis of Cmchi1 *and* Cmchi2

Southern analysis was performed to detect other genes similar to *Cmchi1* or *Cmchi2* in the muskmelon genome. Two hybridization signals were detected when genomic DNA was digested by restriction enzymes *Bam*HI, *Eco*RI or *Hin*dIII and probed with DIG-labelled *Cmchi1* cDNA (Fig. 5A). Hence, at least one additional gene similar to *Cmchi1* may exist in the

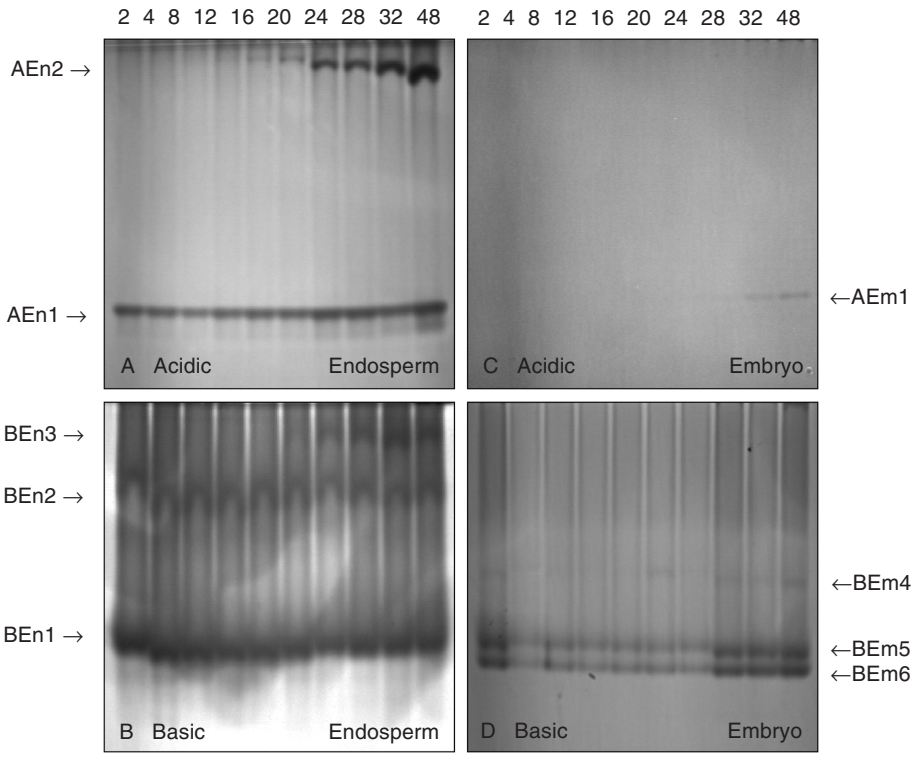

Figure 2. Native-PAGE and activity staining of acidic (A, C) and basic (B, D) chitinase isoforms from muskmelon endosperm (En; A, B) and embryo (Em; C, D) tissue. Seeds were imbibed for 2–48 h and the endosperm and embryo tissues were isolated. Protein extracts (20 µl) from equal fresh weight samples (5 g) were loaded in each lane. Acidic (AEn, AEm) and basic (BEn, BEm) isoforms are marked along the gels.

muskmelon genome, or *Cmchi1* may contain introns harbouring restriction sites. A single hybridization signal was produced when genomic DNA was digested by *Bam*HI, *Eco*RI or *Hin*dIII and probed with DIG-labelled *Cmchi2* cDNA (Fig. 5B), indicating that a single copy of *Cmchi2* is present in the genome.

Expression and induction of Cmchi1 *and* Cmchi2

Expression patterns of both *Cmchi1* and *Cmchi2* were determined during imbibition and after radicle emergence. *Cmchi1* mRNA was first detected in seeds imbibed for 24 h (radicle emergence began at 16 h) and its abundance increased thereafter (Fig. 6A). *Cmchi2* mRNA was detected in seeds imbibed for at least 16 h, and also increased in abundance with time (Fig. 6B). *Cmchi1* and *Cmchi2* mRNAs were also present in root tissue but not in stem, leaf or flower tissues from field-grown muskmelon plants (Fig. 6C, D). In germinated seeds, *Cmchi1* and *Cmchi2* mRNAs were present exclusively in the radicle (Fig. 7).

Salicylic acid (SA) and benzothiadiazole (BTH) induce gene expression associated with systemic acquired resistance (Lawton *et al*., 1995; Görlach *et al*., 1996). When seeds were imbibed in SA or BTH for 8 h, the *Cmchi1* mRNA content did not increase significantly (Fig. 8A). An apparent increase of *Cmchi1* mRNA occurred in seeds imbibed in SA for 16 h. When seeds were imbibed in SA for 24 h, *Cmchi1* mRNA isolated from germinated seeds increased substantially. Treatment with BTH and SA did not increase the expression of *Cmchi2* mRNA relative to controls (Fig. 8B).

Discussion

Several isoforms of chitinase were detected in developing and germinating muskmelon seeds. An acidic chitinase isoform (AD1) present in developing and mature whole fresh seeds had an electrophoretic mobility identical to isoform AEn1 found in the endosperm of seeds within 2 h of imbibition (Figs 1A and 2A, B). As the AD1 samples contained both endosperm and embryo tissues, it is likely that AD1 and AEn1 are the same enzyme, but this would need to be confirmed by more rigorous methods. Similarly, basic chitinase activities were present from 40 DAA

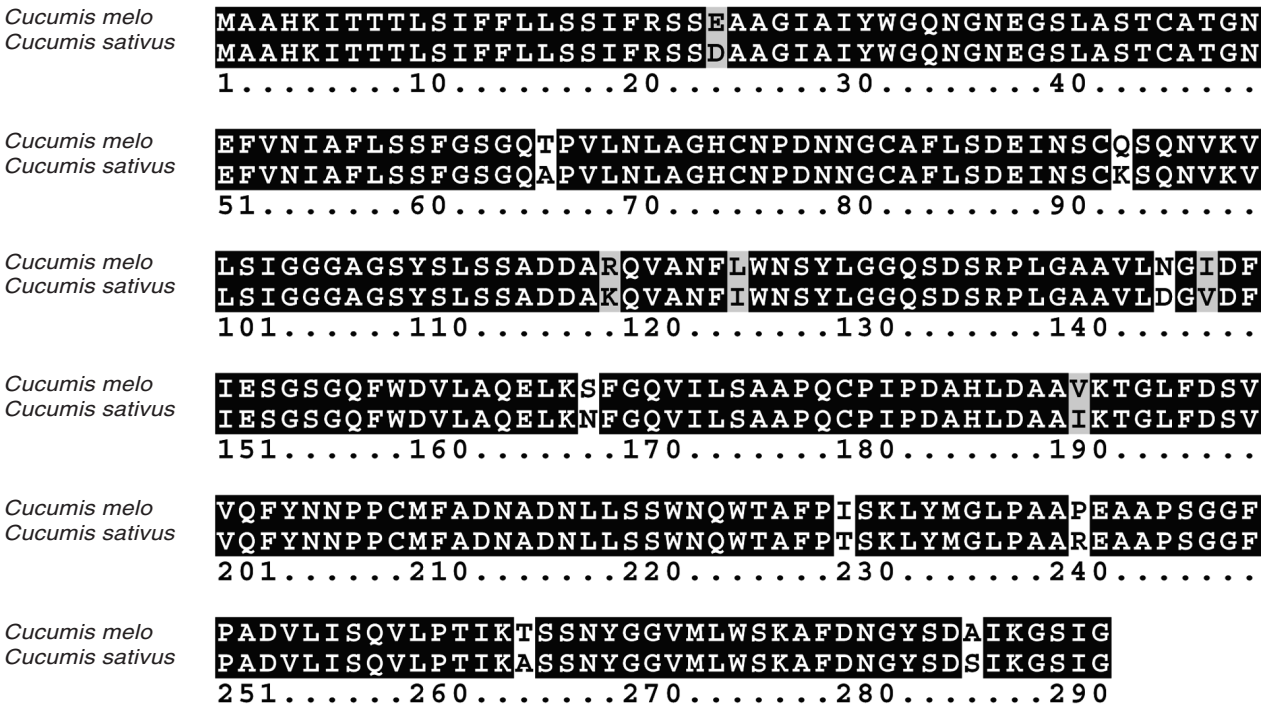

Figure 3. Amino acid sequence alignment of *Cmchi1* (AF241266) against a chitinase from *Cucumis sativus* (P17541). A BLAST (web site: http://www.ncbi.nlm.nih.gov/BLAST/) program (Altschul *et al.*, 1990) search of *Cmchi1* in GenBank produced 95% identity with a cucumber chitinase (Métraux *et al.*, 1989) aligned using ClustalW (Thompson *et al.*, 1994). The alignment was shaded using Boxshade (web site: http://www.ch.embnet.org/software/BOX_form.html) to indicate identical residues in black. The position of the amino acid in the protein is indicated below each sequence.

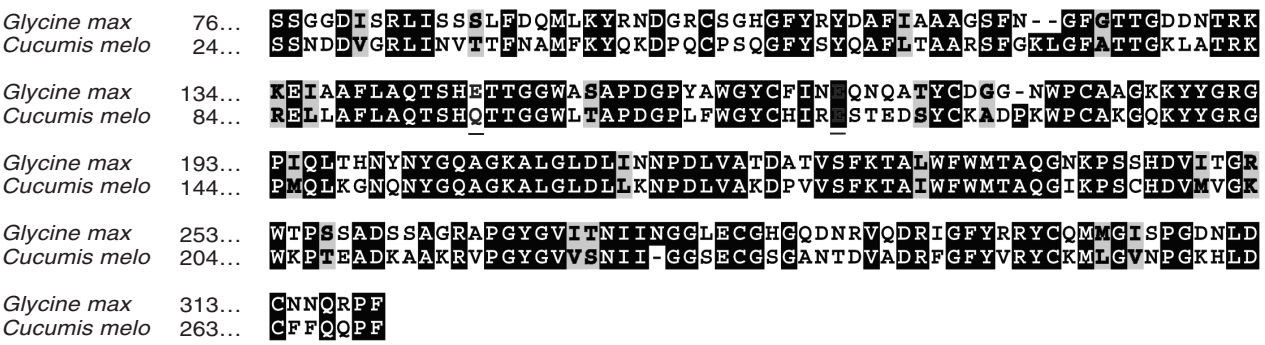

Figure 4. Amino acid sequence alignment of *Cmchi2* (AF241267) against a seed coat chitinase from *Glycine max* (AF202731). A BLAST (web site: http://www.ncbi.nlm.nih.gov/BLAST/) program (Altschul *et al.*, 1990) search of *Cmchi2* in GenBank produced 61% identity with a soybean chitinase (Gijzen *et al.*, 2001) aligned using ClustalW (Thompson *et al.*, 1994). The alignment was shaded using Boxshade (web site: http://www.ch.embnet.org/software/BOX_form.html) to shade conserved residues in black and similar residues in grey. The position of the amino acid in the protein is indicated on the left side. Underlined sequences show conserved Glu residues that occur at the active site. The sequences are offset, in part, because *Cmchi2* lacks a chitinase-binding domain.

during seed development (Fig. 1B) and during imbibition and germination (Fig. 2B, D). Again, it is likely that isoform BD1 during development corresponds to isoform BEn1 during germination and that isoforms BD2 and BD3 correspond to BEm1 and BEm2. In addition, both acidic (AEn2) and basic (BEm1, BEm2) isoforms increased in activity after 20–28 h of imbibition (Fig. 2). Thus, some chitinase isoforms appear to be synthesized during seed development and remain active during germination, while other isoforms are induced in association with, or soon after, radicle emergence.

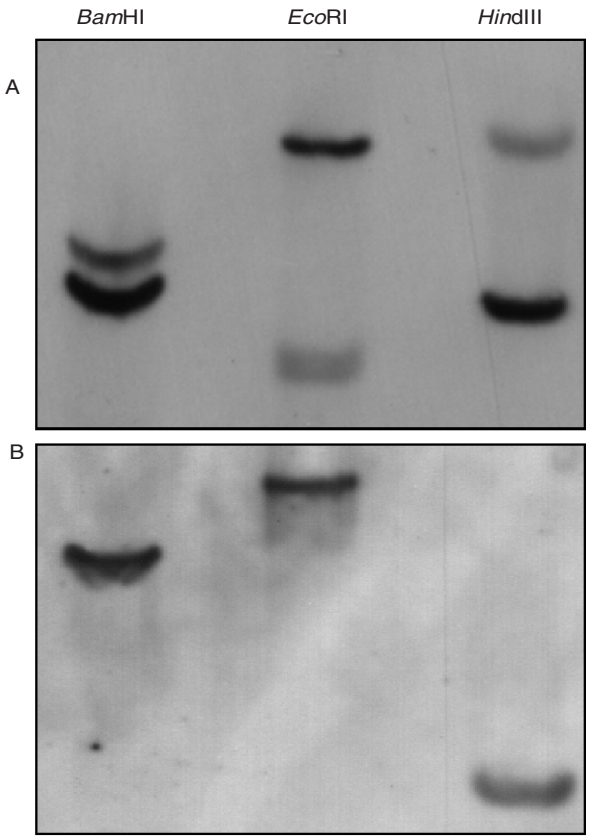

Figure 5. Southern blot of muskmelon genomic DNA hybridized with DIG-labelled full-length cDNAs of *Cmchi1* (A) and *Cmchi2* (B). Genomic DNA isolated from 3-day-old muskmelon seedlings (20 µg/lane) was digested with restriction enzymes *Bam*HI, *Eco*RI and *Hin*dIII. The digested DNA fragments were separated by electrophoresis on a 1.0% agarose gel and denatured. The denatured fragments were transferred to a membrane and probed with DIG-labelled *Cmchi1* cDNA or DIG-labelled *Cmchi2* cDNA.

Two cDNAs encoding putative chitinases were isolated by RT-PCR from developing and germinating seeds. *Cmchi1* shared 95% identity with a class III chitinase from cucumber, suggesting that the two genes are orthologues in related species of *Cucurbitaceae* (Fig. 3; Métraux *et al*., 1989). The cucumber chitinase was expressed in roots and leaves, but expression in seed tissues was not examined (Lawton *et al*., 1994). Unlike the cucumber chitinase, *Cmchi1* was only expressed in root tissue, and the onset of expression in seeds was associated with radicle emergence (Fig. 6C), suggesting that *Cmchi1* may be useful in identifying promoters that are radicle-specific and germination-dependent. A ripening-regulated, putative class III chitinase gene fragment has been cloned from muskmelon fruit (Hadfield *et al*., 2000), but this fragment showed little similarity to *Cmchi1*. A second muskmelon gene encoding a class II chitinase, *Cmchi2*, was also expressed in germinating seeds and roots (Fig. 6B, D). A class I chitinase in soybean that was more highly expressed in the seed coat than in other tissues showed 61% identity to the deduced amino acid sequence of *Cmchi2* (Fig. 4; Gijzen *et al*., 2001). The sequence similarity is not surprising, since class II chitinases are thought to have evolved from class I chitinases through the deletion of the chitin-binding domain (Gomez *et al*., 2002). High-stringency Southern analysis showed one or two copies of *Cmchi1* and a single copy of *Cmchi2* (Fig. 5), consistent with other reports that plant chitinases of class I and III are encoded as single copies or small gene families (Leah *et al*., 1991; Yeboah *et al*., 1998; Gijzen *et al*., 2001). Northern blot analyses indicated that *Cmchi1* and *Cmchi2* were expressed in seeds after germination (Fig. 6), but not in developing seeds (data not shown). Therefore, additional genes other than *Cmchi1* and *Cmchi2* must be responsible for the chitinase activity detected in developing muskmelon seeds (Figs 1, 2).

Gomez *et al*. (2002) have recently reviewed seed chitinases. Seed-specific chitinases are diverse and belong to classes I, II, IV and VII, encoded by *Chia* genes, as well as the more divergent class III forms encoded by *Chib* genes (Gomez *et al*., 2002). As chitin is not a component of plant cell walls, multiple functions for plant chitinases have been proposed, including nodulation, the cleavage of Nod factors, embryogenesis, antifreeze and tissue weakening during radicle emergence (Gomez *et al*., 2002). Possible targets for plant chitinase include arabinogalactan proteins (AGPs), which contain plant-derived substrates for chitinase and are proposed to have multiple roles in plant development, including embryogenesis (Domon *et al*., 2000; Majewska-Sawka and Nothnagel, 2000). However, chitinase activity first appeared in muskmelon seeds at 35 DAA, after the accumulation of maximum dry weight when embryogenesis is essentially complete (Fig. 1; Welbaum and Bradford, 1988).

Chitinase expression in caryopses of barley (*Hordeum* spp.), maize and sorghum was induced by fungal pathogens during development or germination when grain moisture content was high (Swegle *et al*., 1992; Gomez *et al*., 2002). The endosperm tissue in muskmelon seeds protects against fungal attack by limiting electrolyte leakage, due to its semipermeable characteristics, and by putatively acting as a physical barrier to fungal hyphae (Welbaum and Bradford, 1990; Yim and Bradford, 1998). Chitinase in the endosperm may be part of a defence strategy to protect embryos from fungal attack (Fig. 2A, B). Developing muskmelon seeds incubated in Petri plates on filter paper in

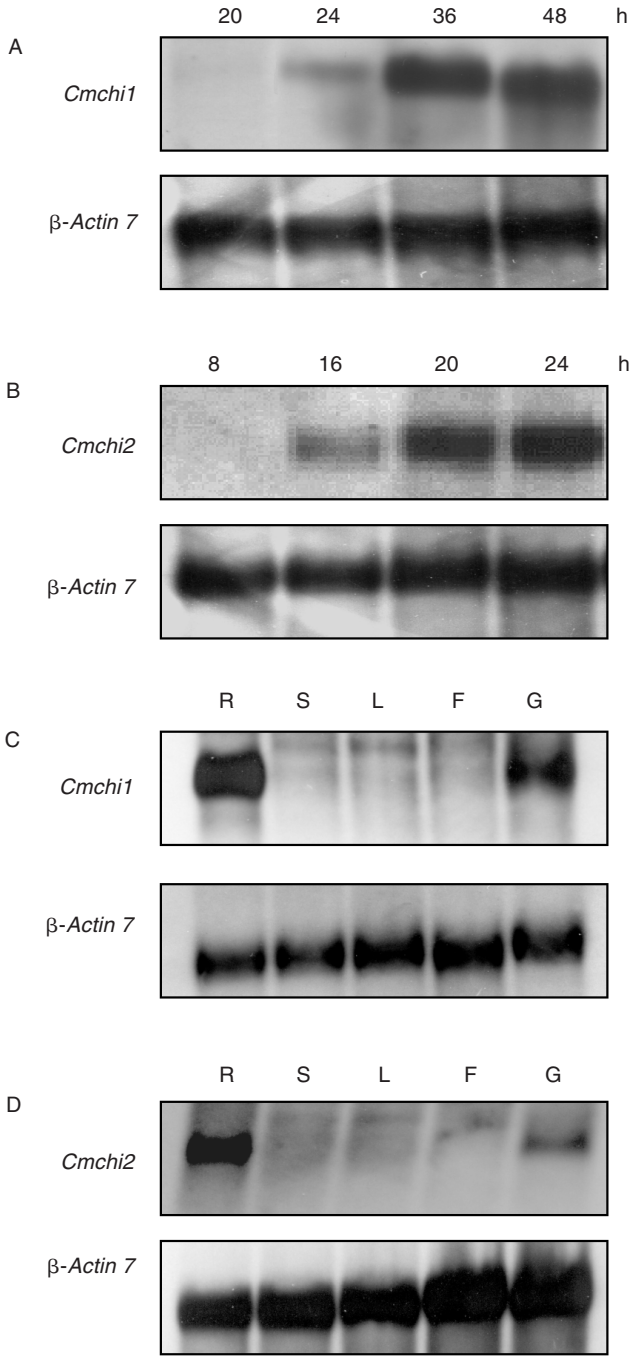

Figure 6. Northern blot of analyses of *Cmchi1* (A, C) and *Cmchi2* (B, D) gene expression in imbibed seeds (A, B) and in various muskmelon tissues (C, D). Poly(A^+) RNA was isolated from seeds imbibed for 8–48 h, as well as from: roots, R; stems, S; leaves, L; flowers, F; and germinated seeds (G). In each panel, the same membrane was stripped and reprobed again with *β-Actin 7* to indicate RNA loading (lower panel).

unsterilized tap water resisted fungal attack longer than stored mature seeds imbibed under the same conditions, suggesting a disease resistance mechanism in developing seeds (data not shown). Once the physical barrier provided by the endosperm is broken during radicle emergence, *Cmchi1* and *Cmchi2* may contribute to protecting the seedling from fungal attack. Such a defensive role for muskmelon chitinase is consistent with the proposed role of embryo- and endosperm-associated chitinases

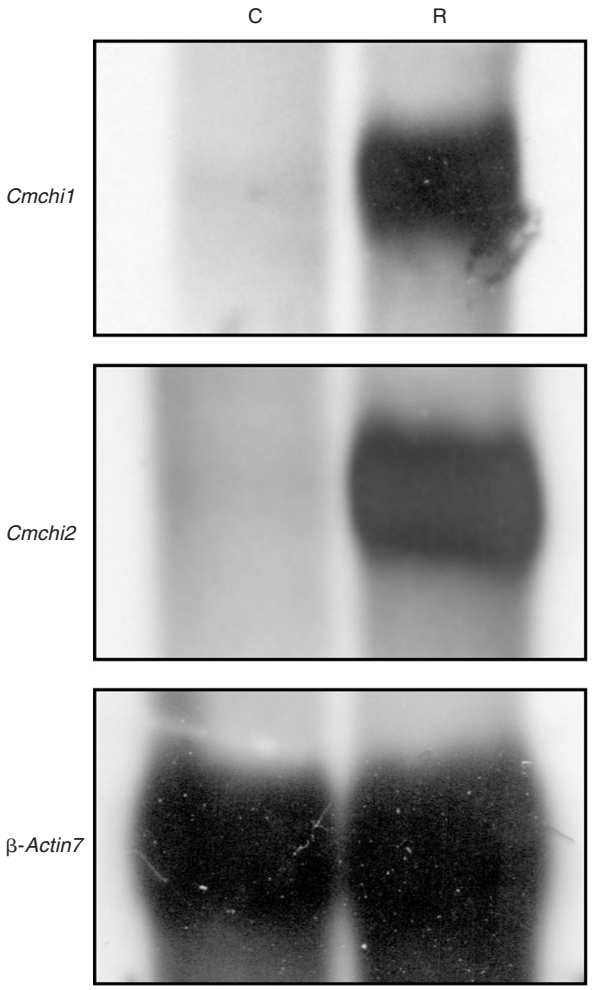

Figure 7. Northern blot analyses of *Cmchi1* and *Cmchi2* gene expression in radicle, cotyledon and endosperm tissues. Muskmelon seeds were imbibed for 24 h and dissected into the radicle (R) and cotyledons, including endosperm tissue (C), for RNA extraction. Membranes were reprobed with *β-Actin 7* to indicate RNA loading.

in seeds of other species (Wu *et al*., 2001; Gomez *et al*., 2002).

Although purified chitinases from pea and other species have inhibited fungal growth *in vitro* (Mauch *et al*., 1988; Gomez *et al*., 2002), crude protein extracts from muskmelon seeds failed to inhibit growth of *Tricoderme viride* and *Rhizoctonia solani* (data not shown), which contain chitin in their cell walls. The chitinase concentration in crude extracts may have been insufficient to be effective, or other factors may be required. Chitinases act synergistically with other enzymes such as β-1,3-glucanase (Leubner-Metzger and Meins, 1999; Neuhaus, 1999; Gomez *et al*., 2002), but β-1,3-glucanase activity was not detected in muskmelon seeds (data not shown). Unfortunately, purified recombinant Cmchi1 protein expressed in bacteria did not show enzymatic activity with glycol chitin as substrate (data not shown), preventing direct investigation of its potential defensive role in fungal inhibition assays.

Chitinase expression is correlated with systemic acquired resistance (SAR) in plants such as cucumber and tobacco (Métraux *et al*., 1989; Shinshi *et al*., 1990; Lawton *et al*., 1994). SAR can be induced in higher plants by compounds such as SA or its analogue, BTH (Uknes *et al*., 1992; Görlach *et al*., 1996). Previous studies have shown that seed chitinases are produced constitutively or in response to fungi, but relatively little is known about whether chitinase expression in seeds can be induced as part of a SAR response. Several studies have shown that acidic chitinases, but not basic chitinases, were induced by SA (Brederode *et al*., 1991; Buchter *et al*., 1997; Ancillo *et al*., 1999). In cucumber, the expression of a class III chitinase increased tenfold following application of SA (Lawton *et al*., 1994). In our study, imbibing seeds in 1 mM SA or 0.3 mM BTH did not increase the expression of *Cmchi2*, a basic class II chitinase (Fig. 8B). This indicates that a SA-independent pathway may lead to the expression of class II chitinase genes such as *Cmchi2*. Application of SA or BTH increased *Cmchi1* (class III) mRNA content in muskmelon seeds during imbibition, and particularly after radicle emergence (Fig. 8A). This suggests that *Cmchi1*

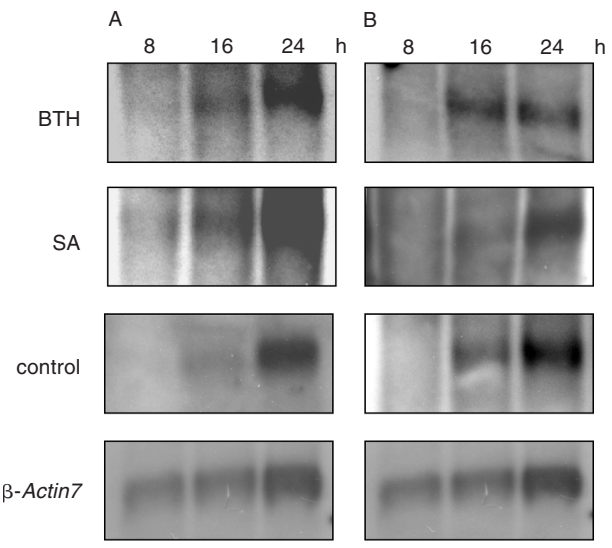

Figure 8. Northern blot analysis of *Cmchi1* (A) and *Cmchi2* (B) gene expression in muskmelon seeds imbibed in water (control), salicylic acid (SA, 1 mM) or benzothiadiazole (BTH, 0.3 mM). Membranes were stripped and reprobed with *β-Actin 7* to indicate RNA loading.

expression could be part of a SAR response in newly germinated seeds.

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