An Acidic Class III Chitinase in Sugar Beet: Induction by *Cercospora beticola*, Characterization, and Expression in Transgenic Tobacco Plants

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An acidic chitinase (SE) was found to accumulate in leaves of sugar beet (*Beta vulgaris*) during infection with *Cercospora beticola*. Two isoforms, SE1 and SE2, with MW of 29 kDa and pI of approximately 3.0 were purified to homogeneity. SE2 is an endochitinase that also exhibits exochitinase activity, i.e., it is capable of hydrolyzing chitooligosaccharides, including chitobiose, into *N*-acetylglucosamine. Partial amino acid sequence data for SE2 were used to obtain a cDNA clone by polymerase chain reaction. The clone was used to isolate a cDNA clone encoding SE2. The deduced amino acid sequence for SE2 is 58–67% identical to the class III chitinases from cucumber, *Arabidopsis,* and tobacco. A transient induction of SE2 mRNA during the early stages of infection with *C. beticola* is much stronger in tolerant plants than in susceptible plants. Transgenic tobacco (*Nicotiana benthamiana*) plants constitutively accumulate SE2 protein in the intercellular space of their leaves. In a preliminary infection experiment, the transgenic plants did not show increase in resistance against *C. nicotianae.*

Additional keywords: antifungal, cDNA sequence.

The response of plants to infection with pathogens is complex and includes the synthesis of a number of so-called pathogenesis-related (PR) proteins thought to be involved in active defense against the invading pathogens (for review see Boller 1987; Collinge and Slusarenko 1987; Dixon and Harrison 1990). For some classes of PR proteins, no function has been discovered to date. However, for the hydrolytic chitinases (PR-3 group) and β-1,3-glucanases (PR-2 group), the cell walls of many phytopathogenic fungi containing large amounts of chitin and β-1,3-glucan are the obvious target (Abeles *et al.* 1971; Molano *et al.* 1979; Wessels and Sietsma 1981). Furthermore, these enzymes have been shown to inhibit the growth of filamentous fungi *in vitro* (Broekaert *et al.* 1988; Broglie *et al.* 1991; Mauch *et al.* 1988; Schlumbaum *et al.* 1986). For these reasons, considerable effort has been aimed at isolating and characterizing these plant hydrolases to evaluate their potential for improving disease resistance of plants against fungi (Collinge *et al.* 1993).

One of the most serious diseases in sugar beet (*Beta vulgaris* L.) is the leaf spot disease, caused by the fungus *Cercospora beticola* (Sacc.). The only way to control the disease at present is by intensive spraying with fungicides. An alternative means of control may be by increasing the expression level of one or more PR proteins by genetic engineering. We are analyzing the natural defense responses in sugar beet expressed during infection with *C. beticola.* We have studied the induction of chitinases and β-1,3-glucanases in leaves following infection with this specific pathogen (Mikkelsen *et al.* 1992). Here, we report the purification and characterization of an acidic chitinase that is strongly induced during infection with *C. beticola.* Two isozymes have been purified to homogeneity. We also report the isolation and sequencing of a gene encoding one of the isoforms that shows significant structural similarity to the class III chitinases. Finally, with the aim of testing the *in vivo* antifungal potential of the chitinase against *Cercospora* spp., transgenic tobacco plants constitutively expressing this chitinase have been constructed and subsequently challenged with *C. nicotianae.*

RESULTS

Purification and characterization of an acidic chitinase from sugar beet leaves infected with *C. beticola.*

Purification: An acidic chitinase, SE, was purified by two consecutive anion-exchange chromatography steps followed by chromatofocusing. The former steps were necessary to remove considerable amounts of polyphenols and other impurities. In the final purification step, three protein peaks (1–3 in Fig. 1) with chitinase activity were eluted from the Mono P anion exchange column. Examination by silver staining after SDS-PAGE revealed that peaks 1 and 2 (SE1 and SE2) were comprised of single proteins with MW of 29 kDa (Fig. 2A). In contrast, several protein bands were observed for peak 3 (results not shown). By IEF the pIs of the SE1-3 proteins were found to be approximately 3.0 (results not shown).

Immunological characterization: A polyclonal antibody raised against the SE2 isozyme reacted with both chitinase

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Nucleotide and/or amino acid sequence data have been submitted to GenBank, EMBL, and DDBJ as accession number J03714.

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isozymes SE1 and SE2 when examined by immunoblotting after SDS-PAGE (Fig. 2B), showing that they are serologically related. In addition, SE isoforms in crude extracts (Fig. 3), and after various purification steps, could be recognized by an antibody raised against the basic sugar beet chitinase 4, belonging to the class IV chitinases (Collinge *et al.* 1993; Mikkelsen *et al.* 1992). In addition, the purified SE1 and SE2 isozymes were recognized by the chitinase 4 antibody, and vice versa: The SE2 antibody recognized chitinase 4 (results not shown), demonstrating that the acidic SE chitinase is serologically related to the basic chitinase 4. In contrast, no serological relationship was found between SE2 and the basic class I sugar beet chitinase 2.

Hydrolytic activities and chitin binding: The reaction products liberated by the SE2 chitinase from [^{3}H]-chitin were separated by HPLC on a silica column and quantified on the basis of UV absorbance and radioactivity (Fig. 4; Table 1). When the SE2 isozyme was incubated with [^{3}H]-chitin for 1 hr, the major product of the enzymatic hydrolysis was the dimer, chitobiose. In addition, some monomer, trimer, and small amounts of tetra- and pentamer were present (Fig. 4A). Incubation for 6 hr increased the amount of monomer and dimer, whereas the amounts of trimer and higher oligomers decreased correspondingly (Fig. 4B). These results show that SE2 is an endochitinase, i.e., the hydrolytic action on chitin releases dimer and higher oligomers. In addition, SE2 is able to hydrolyze the dimer and small oligomers to release monomer. This was found by incubating the reaction products released from [^{3}H]-chitin after 24 hr of hydrolysis for additional 12 hr with SE2. Virtually no trimer and higher oligomers were left, and about 70% of the final reaction product was detected as monomer (Fig. 4C).

No lysozyme activity could be demonstrated for SE2 when assayed at pH 4–9. Furthermore, no adsorbtion of SE2 to the chitin affinity column was observed at pH 2.5–10.0. The stability of SE2, as determined by the specific chitinase activity, was not affected by exposure to the various pH regimes used.

Induction of SE after infection with ***C. beticola:*** Fourteen days after inoculation with *C. beticola*, the SE protein in sugar beet leaves was found to be induced when analyzed by Western blotting using the chitinase 4 antibody. In leaf homogenates from infected plants, two distinct protein

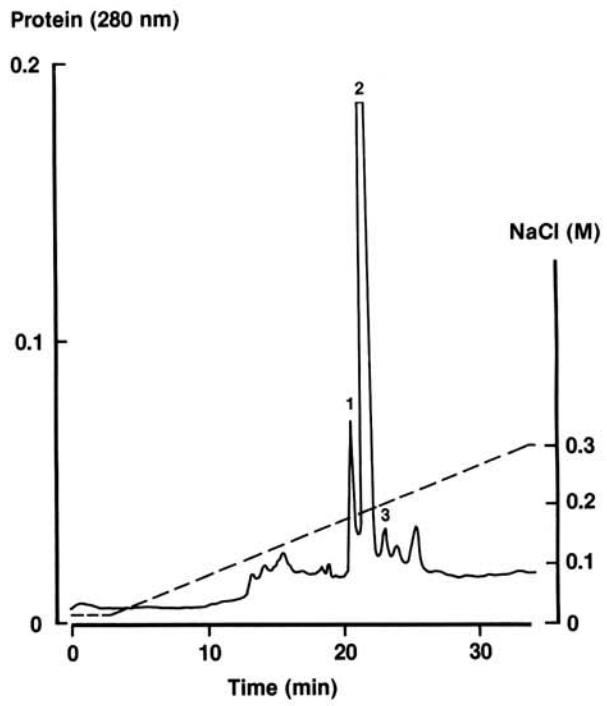


Fig. 1. Separation of SE isozymes from *Cercospora*-infected sugar beet leaves by fast protein liquid chromatoraphy (FPLC) on an anion-exchange Mono P column. The proteins were eluted with a linear gradient of 0–0.3 M NaCl in 25 mM Bis-Tris, pH 7.0, as indicated. Flow rate was 1.0 ml/min.

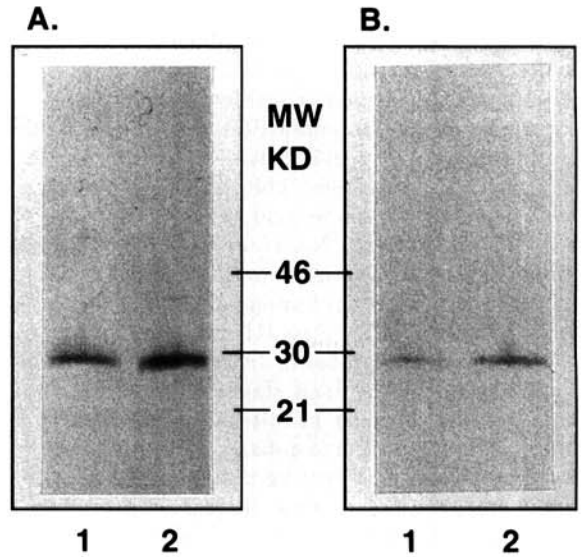


Fig. 2. Analysis of the acidic chitinase isozymes SE1 and SE2 by SDS-PAGE. **A,** Silver-stained 10–15% gradient Phast Gel; **B,** Immunoblotting. After separation on a 10–15% gradient gel the proteins were electroblotted onto a nitrocellulose membrane and probed with antibody against chitinase isozyme SE2. Lane 1: SE1, lane 2: SE2 (50 ng of protein loaded in each lane).

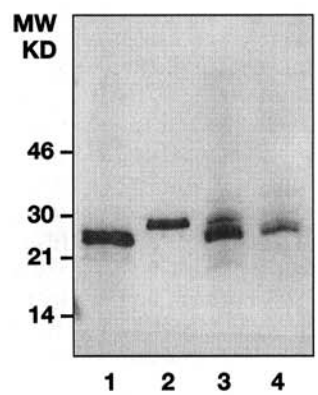
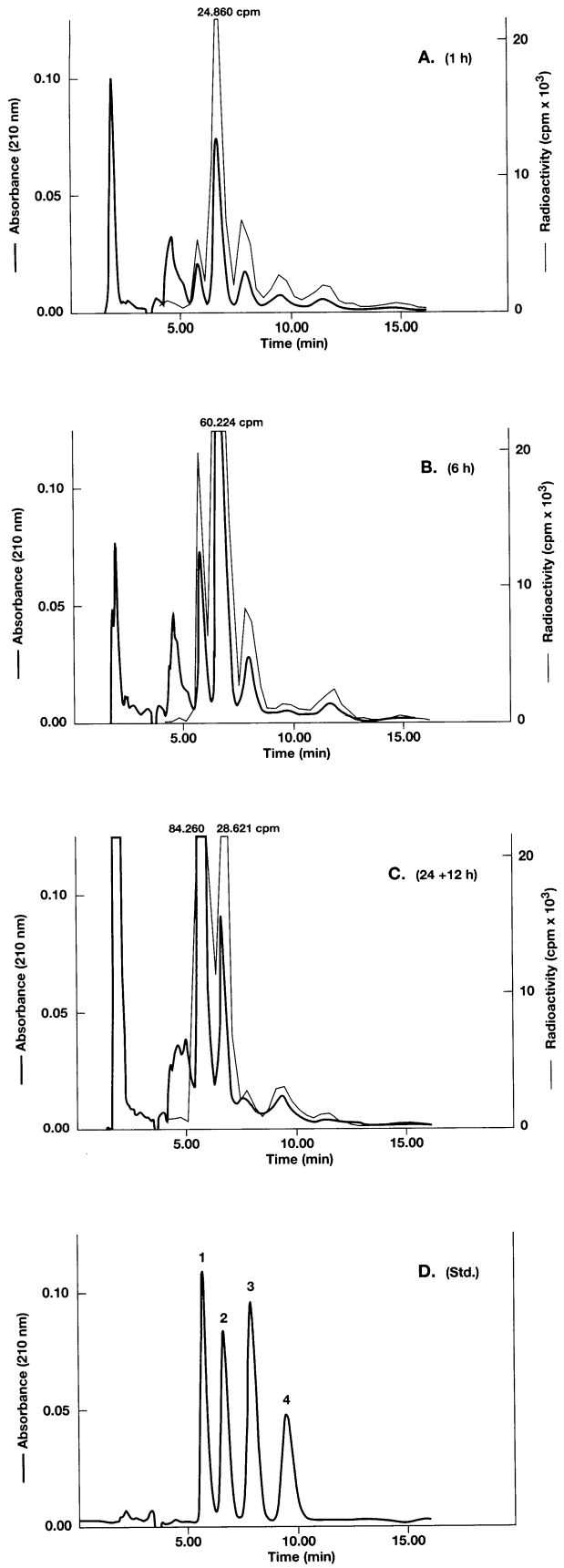


Fig. 3. Immunoblot probed with antibody against chitinase 4. Crude leaf homogenates from sugar beet (*Beta vulgaris* 'Monova') were fractionated by tricine SDS-PAGE (16.5% gel) and electroblotted onto a nitrocellulose membrane. Lane 1, purified chitinase 4 (100 ng); lane 2, purified SE2 (100 ng); lane 3, *Cercospora*-infected sugar beet, 14 days after inoculation (20 μg of total protein); lane 4, uninfected, 14 days after mock inoculation (20 μg).



bands with MW's of ~29 and 26 kDa, corresponding to SE and chitinase 4, respectively, were recognized by the antibody (Fig. 3, lane 3). In mock-inoculated control plants, no SE protein could be detected (Fig. 3, lane 4).

Amino acid sequence.

After tryptic digestion of the SE2 isozyme, six peptides were rechromatographed on a Devosil C18 column and subjected to amino acid sequencing. In addition, the N-terminal amino acid sequence of SE2 was determined. These amino acid sequences covering a total of 87 residues are marked in the amino sequence shown in Figure 5.

Isolation and characterization of the SE cDNA.

The oligonucleotide 270 (5′-CCAAGCTTGAATT-C$(T)_{20}$-3′), comprising two restriction sites (*Hin*dIII and *Eco*RI) followed by an oligo-dT, was used as "downstream" primer both in reverse transcription and in the two successive PCR amplification steps. Two degenerated "upstream" primers were chosen on the basis of the amino acid sequences of the tryptic peptides. Primer KB7 (5′- GACTCT-AGAGAA(TC)CC(GA)CC(GA)TG(TC)CA(AG)TA(TC)GA(TC)AC-3′), extended with 10 bases containing an *Xba*I site, was used in the first PCR amplification, and KB9 (5′-GGA-GGATCCCC(GA)GC(GATC)AA(TC)CA(GA)AT(ACT)TT-3′), supplied with a *Bam*HI site, was used in the second PCR amplification. The positions of the two upstream primers in the SE cDNA are marked in Figure 5. After the second PCR amplification, one distinct DNA product of 251 bp was obtained. The fragment was cloned into a pUC19 plasmid and sequenced. The fragment, identical to nucleotides 711–962 of the DNA sequence shown in Figure 5, was used to screen 1×10^6 plaques from the λ-ZAP II cDNA library. Twenty-three clones hybridized with the PCR fragment at high stringency. Following plaque purification, the 23 clones were converted into plasmids by *in vivo* excision, and the sizes of the inserts were determined. The largest clone, SE-22, was sequenced in both directions. It contained 1,070 bp and corresponded to nucleotides 37–1106 of the cDNA in Figure 5. By re-screening the λ-ZAP II library with a 136-bp *Eco*RI-*Kpn*I fragment from the 5′ end of SE-22 (nucleotides 37–172), one clone (SE-5′) containing the sequence encoding the N-terminus of the mature SE protein as well as the entire N-terminal signal peptide was obtained.

The 1,106-bp SE cDNA (SE-5′/SE-22) obtained exhibits a 5′ noncoding region of 17 bp, an ATG initiation codon at position 18 followed by an open reading frame of 879 bp. The stop codon (TAA at position 897) is followed by a 3′ noncoding region of 202 bp containing a poly-adenylation site at position 934. The derived amino acid

Fig. 4. Analysis of hydrolysis products. The reaction products following hydrolytic action of the SE2 chitinase (10 μg) on insoluble [^{3}H]-labeled chitin (200 μg) were separated by HPLC on a YMC-Pack silicia column. Elution of the chito-oligosaccharides was affected with water/acetonitrile (71.5:28.5, v/v). Flow rate was 0.8 ml/min. **A, B,** Incubation for 1 and 6 hr, respectively. **C,** The 24-hr reaction products were incubated for additional 12 hr with fresh SE2 enzyme (5 μg). **D,** Chito-oligosaccharide standards; mono-, di-, tri- and tetramer (1–4; 15 μg each).

sequence of the coding region contains a N-terminal signal peptide of 25 amino acid residues and a functional domain of 268 amino acid residues (Fig. 5). The deduced amino acid sequence reveals 100% identity with the sequences of the six tryptic peptides and the N-terminal sequence of the native SE2 protein. Furthermore, the MW of 28 kDa and pI of 3.8 calculated for the functional domain, using the algorithm of PC/Gene (IntelliGenetics Inc., CA), are in accordance with the MW of 29 kDa and pI of approximately 3 for SE2 estimated from polyacrylamide gel electrophoresis. These data confirm that the cDNA clone obtained encodes the SE2 polypeptide purified by the chromatographic procedure described above.

Comparison with other chitinases.

The derived amino acid sequence of SE2 exhibits significant similarity to chitinases from *Cucumis sativus* (Metraux *et al.* 1989), *Arabidopsis thaliana* (Samac *et al.* 1990), and *Nicotiana tabacum* (Lawton *et al.* 1992) belonging to the chitinase class III as defined by Shinshi *et al.* (1990) (Fig. 6). The mature SE2 protein shows 66% identity with the acidic chitinase from *C. sativus* and 61% identity with the acidic one from *A. thaliana*. The identity of SE2 with the acidic and the basic chitinases from *N. tabacum* is 67 and 58%, respectively. An acidic class III chitinase from chickpea (*Cicer arietinum*) has recently been described by Vogelsang and Barz (1993). The N-terminal sequence of 30 residues obtained for this chitinase is 53% identical to the N-terminus of SE2. No significant similarity was found to plant chitinases of class I, II, and IV.

Induction of SE2 mRNA.

A clear difference in the accumulation of SE2 mRNA was observed between the *Cercospora*-susceptible (Monova) and the tolerant (+Tol) sugar beet cultivar during early stages of infection as shown for one experiment in Figure 7: In both cultivars, a temporary increase in SE2 mRNA level was observed 9 days after inoculation with *C. beticola*; however, the level in the tolerant cultivar was considerably higher. At day 10, no SE2 mRNA was detected. A second induction of mRNA, starting 12 days after inoculation, was observed in both cultivars. At this time, the expression was higher in the susceptible plants. In mock-inoculated control plants, no SE2 mRNA was detected at any time during the experiment in either of the cultivars. This suggests that induction of SE chitinase occurs mainly at the transcriptional level. Two successive repetitions of the experiment gave the same induction pattern: a 1-day expression of SE2 mRNA at day 8 or 9 and a second prolonged induction starting 12–14 days after inoculation. In both experiments, the early induction was markedly stronger in the tolerant plants. In one experiment, however, the late induction in the tolerant cultivar was almost as strong as in the susceptible cultivar.

Genomic diversity.

Blots of genomic sugar beet DNA from cvs. Monova and +Tol were probed with the SE2 cDNA followed by a high-stringency wash. The genomic DNA had been digested with *Xba*I or *Bam*HI, enzymes for which no recognition sites are present within the SE2 cDNA sequence. Two bands were observed in the *Xba*I digest for both cultivars. In the *Bam*HI digests only one intense band was found in +Tol, whereas an additional low molecular weight band was present in Monova (Fig. 8). These results indicate that only one or a few genes in the sugar beet genome encode SE chitinase and could be explained by a single gene with at least one intron in which there is an *Xba*I site. Polymorphism exists between the two cultivars, which is demonstrated by the difference in band sizes of the *Xba*I digests and by the presence of the low molecular weight band in the *Bam*HI digest in Monova.

Transgenic tobacco plants: Expression and localization of SE2 protein, resistance against *C. nicotianae*.

It was decided to test whether tobacco plants exhibiting a high constitutive extracellular level of the SE chitinase are more resistant to fungal attack. Tobacco was chosen, as transformation and regeneration of this plant species is less cumbersome and time consuming than sugar beet. Moreover, the infection biology of *C. nicotianae* in tobacco is similar to that of *C. beticola* in sugar beet. Finally, as tobacco contains no chitinases that are recognized by the chitinase 4 antibody, the risk of "interference" with

Table 1. SE2 reaction products[a]

		1 h	6 h	24 + 12 h
Monomer				
Peak area[b]	(%)	570 (11)	2,509 (23)	10,482 (71)
Radioactivity[c]	(%)	7,821 (10)	27,099 (17)	121,380 (66)
Dimer				
Peak area[b]	(%)	3,235 (61)	6,566 (59)	3,251 (22)
Radioactivity[c]	(%)	41,837 (52)	98,429 (61)	43,970 (24)
Trimer				
Peak area[b]	(%)	800 (15)	1,408 (13)	227 (2)
Radioactivity[c]	(%)	13,912 (19)	21,193 (13)	3,814 (2)
Tetramer				
Peak area[b]	(%)	346 (7)	70 (1)	589 (4)
Radioactivity[c]	(%)	8,625 (11)	5,482 (3)	11,724 (6)
Pentamer				
Peak area[b]	(%)	314 (6)	525 (5)	275 (2)
Radioactivity[c]	(%)	7,486 (9)	8,897 (6)	3,990 (2)

[a] The reaction products shown in Fig. 4 are quantified on the basis of peak area and collected radioactivity.
[b] 10^{-8} absorbance units × minute.
[c] cpm.

native induced isozymes is eliminated.

The cDNA encoding the SE2 polypeptide was introduced into the plant transformation vector pBKL4 as illustrated in Figure 9 and summarized below. A full-length SE2 cDNA was constructed by combining a *Eco*RI-*Kpn*I fragment from the clone SE-5′ containing the 5′ end of the SE2 cDNA with a *Kpn*I-*Hin*dIII fragment of the clone SE-22 comprising the rest of the gene in the cloning vector pUC19. The SE2 gene was subcloned into the *Sma*I site of pPS48 as a *Eco*RI-*Hin*dIII fragment (filled in with

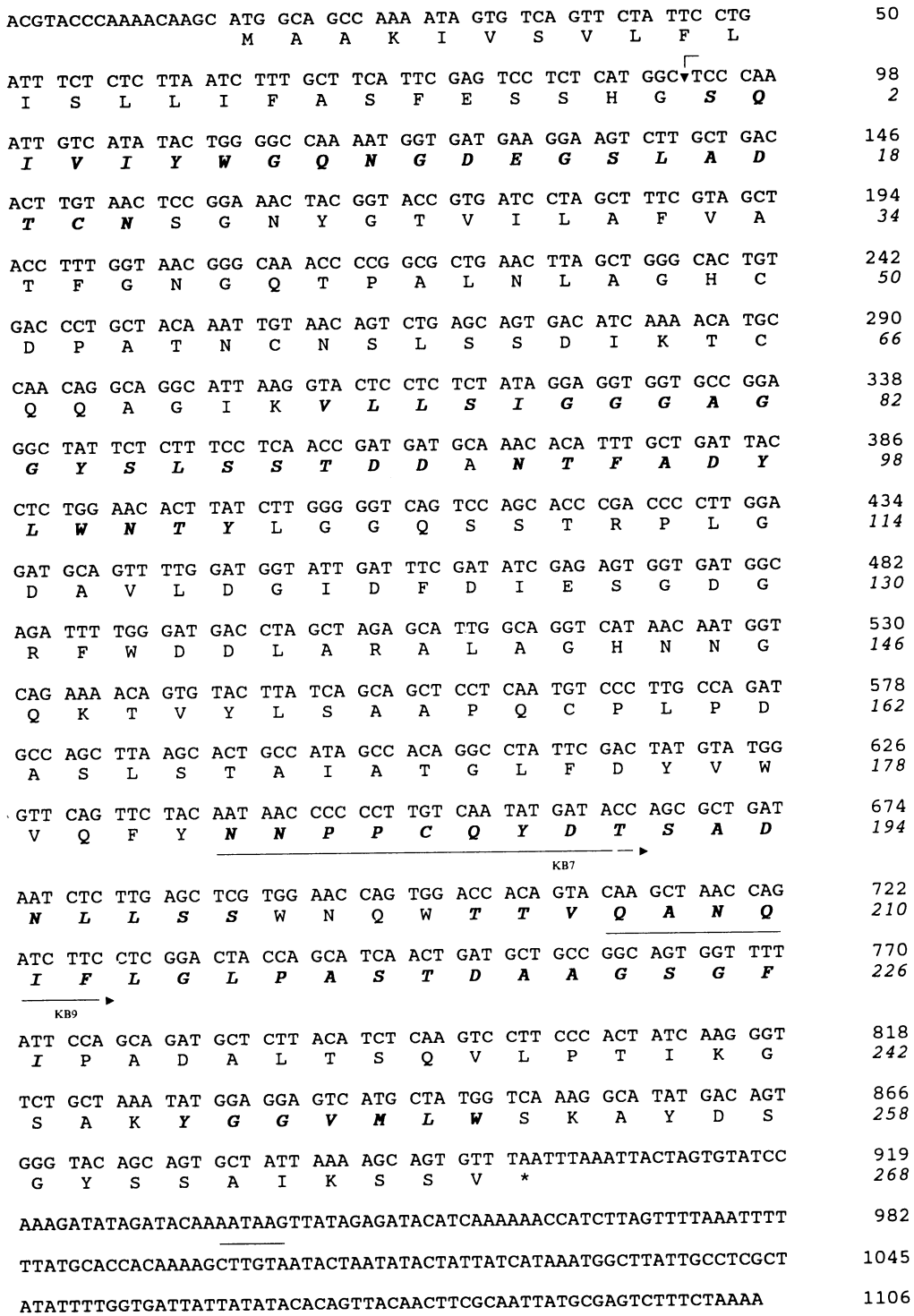


Fig. 5. cDNA sequence and deduced amino acid sequence of the acidic sugar beet chitinase SE2. Arrow indicates the putative signal peptide cleavage site. The stop codon is marked by an asterisk and the putative polyadenylation site is underlined. Peptide sequences are indicated by bold letters in italics. The positions of the PCR primers, KB7 and KB9, are indicated by underlining, with arrowheads indicating polarity 5′ to 3′.

Klenow polymerase). Finally, the SE2 gene, now supplied with an enhanced 35S promoter and a 35S terminator, was cloned into the *Hin*dIII site of pBKL4. The correctness of the vector construct was verified by restriction enzyme analysis and partial DNA sequencing.

The expression level of the SE2 protein in transgenic *N. benthamiana* plants was analyzed by immunoblotting of leaf homogenates fractionated by SDS-PAGE. The protein blots were probed with chitinase 4 antibody. As shown for three plants in Fig. 10 (lanes 2–4), a single distinct protein band, corresponding in MW to the SE2 protein purified from sugar beet leaves (Fig. 10, lane 1), was detected in all of 10 tested primary transformants showing high β-glucuronidase activity. In nontransgenic tobacco plants (data not shown) and in transgenic control plants, containing the NPTII and GUS genes only (Fig. 10, lane 5), no protein was detected by the chitinase 4 antibody. Thus, expression of the SE2 protein was obtained in tobacco leaves when the gene was regulated by the enhanced 35S promoter. Based on visual comparisons of band intensities on immunoblots, the levels of SE2 protein in the transgenic tobacco leaves were comparable to those in sugar beet leaves following infection with *C. beticola*.

To analyze the subcellular localization of the SE2 protein expressed in the transgenic plants, intercellular washing fluids (IWF) from mature leaves of the 10 transformants were pooled and probed with the chitinase 4 antibody. A strong reaction with a 29-kDa protein band in the IWF (Fig. 10, lane 6) and lack of recognition in the extract of leaves homogenated after removal of the IWF (Fig.

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SE2              SQIVIYWGQNGDEGSLADTCNSGNYGTVILAFVATFGNGQTPALNLAGHC   50
C. sativus       AG·A·····N·····S··AT···EF·NI··LSS··S··A·V·····      50
A. thaliana       G·A·····N··N·SA··AT·R·AY·NV··LVK·····E·····       49
N. tabacum (b)   GD·VV····DVG··K·I·····L·NI·NI··LSS···F···K·····      50
N. tabacum (a)   GD·V·····N·····ATN··AI·NI··LVV·····N·V·····          50

SE2              DPATN-CNSLSSDIKTCQQAGIKVLLSIGGGAGGYSLSSTDDANTFADYL   99
C. sativus       N·DN·G·AF··DEINS·KSQNV·····S·····A···KQV·NFI        100
A. thaliana      N··A·T·THFG·QV·D··SR····M··L···I·N··IG·RE··KVI····   99
N. tabacum (b)   E·SSGG·QQ·TKASRH··SI···IM·····TPT·T···V···RQV····    100
N. tabacum (a)   ··NAGA·TG··N··RA··NQ····M··L·····S·F···A···RNV·N··   100

SE2              WNTYLQQQSSTRPLGDAVLDGIDFDIESGDGRFWDDLARALAGHNNGQKT   149
C. sativus       ··S··GG··DS····A·····V·····S·Q···V··QE·KNFGQ----     146
A. thaliana      ··NF·GGK··S·····N··L·SPQH·····T·SKFSHRGRK            149
N. tabacum (b)   ··NF·GG···F·····L·-QPHYIA···R·SE·GQQG·K              149
N. tabacum (a)   ··N··GG··N·····G·TTQH··E··KT·SQFSQQ-RK               149

SE2              VYLSAAPQCPLPDASLSTAIATGLFDYVWVQFYNNPPCQYDT-SADNLLS   198
C. sativus       ·I·····I···H·DA··K·····S·····MFAD-N·····             195
A. thaliana      I··TG·····F··RLMGS·LN·KR·····I·····S·SSGNTQ··FD      199
N. tabacum (b)   L··T·····F··KL·NG·LQ·····E·EFMSN··-E·FKR             198
N. tabacum (a)   ···T·····F··TW·NG·LS·····SGG·····KN                  199

SE2              SWNQWTT-VQANQIFLGLPASTDAA-GSGFIPADALTSQVLPTIKGSAKY   246
C. sativus       ·····A-FPTSKLYM····ARE··PSG·····V·I·····A·SN·       244
A. thaliana      ···K···SIA·QKF·····APE··-D··Y··P·V·T··I···L·K·R·`   248
N. tabacum (b)   R·····-SIPAKKLYI····AKT··-·N·Y··KQV·M·····FL···S··  246
N. tabacum (a)   Y····N-AI··GK·····AQG··-·····SDV·V·····L·N··P··     246

SE2              GGVMLW-SKAYD--SGYSSAIKSSV                           268
C. sativus       ·····-···F·--N···DS··G·IG                           267
A. thaliana      ·····-··FW·DKN····S·LAS·                            272
N. tabacum (b)   ·····NRKFDVQC-·····RGA·                             270
N. tabacum (a)   ·····-··F··N--·····AN·                              268
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Fig. 6. Comparison of the amino acid sequence of sugar beet chitinase SE with acidic class III chitinases from *Cucumis sativus* (Metraux *et al.* 1989) and *Arabidopsis thaliana* (Samac *et al.* 1990) and a basic (b) and acidic (a) class III chitinase from *Nicotiana tabacum* (Lawton *et al.* 1992). Amino acids identical to those in SE2 are indicated by dots.

10, lane 7) indicate that the SE2 chitinase is excreted into the intercellular space.

Ten micro-propagated plants from each of the 10 genotypes exhibiting good extracellular levels of SE2 accumulation were evaluated for resistance against *C. nicotianae.* Compared to control plants, no differences in time of appearance or severity of symptoms were observed.

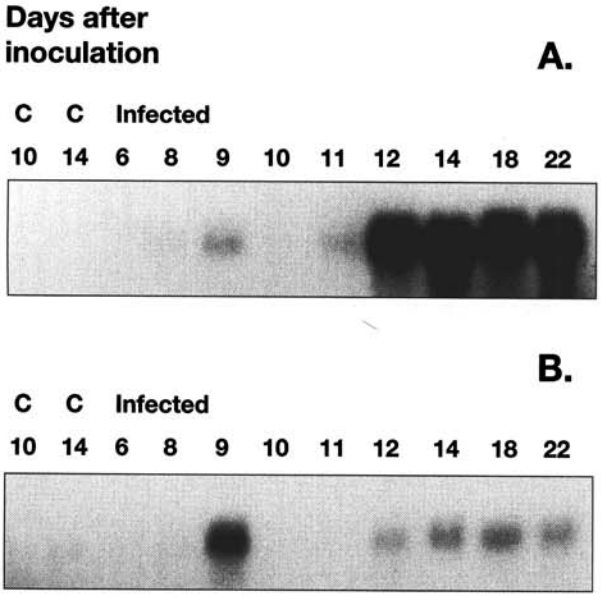

Fig. 7. Northern blot hybridization analysis. Induction of SE2 chitinase mRNA in sugar beet leaves of cv. Monova (susceptible, **A**) and cv. +Tol (tolerant, **B**) during infection with *Cercospora beticola.* Aliquots of 15 μg of total RNA was applied to the gel. C: Control plants.

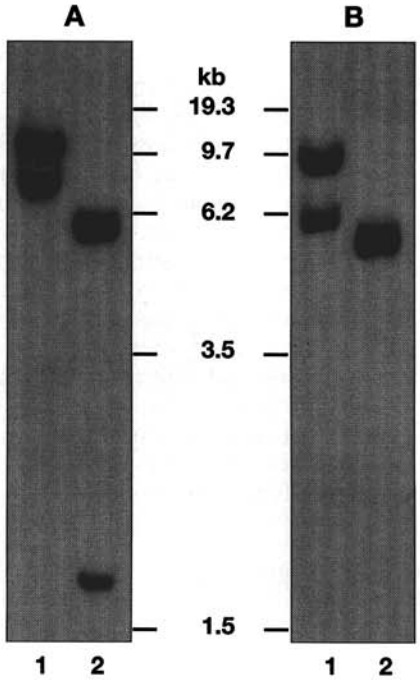

Fig. 8. Southern blot hybridization analysis. Genomic sugar beet DNA (20 μg) from cvs. Monova (A) and +Tol (B) was digested with *Xba*I (1) and *Bam*HI (2) and hybridized with the SE2 chitinase cDNA clone. The blots were washed at high stringency.

DISCUSSION

The high amino acid sequence identity of SE2 with the class III chitinases from cucumber, *Arabidopsis,* and tobacco (Fig. 6) shows that SE2 belongs to this class. The lack of binding of SE2 to the chitin affinity column is in accordance with the class III chitinase from cucumber, showing only very low affinity for a chitin column (Metraux and Boller 1986). So far, all plant chitinases that have been characterized for their reaction products are endochitinases releasing mainly dimer, trimer, and tetramer during hydrolytic action on chitin. Class I and II endochitinases have been isolated from a number of plant species and tissues, e.g., bean and pea leaves, barley grain, carrot cell cultures, melon plants, wheat germ, and wheat leaves (Boller 1983 *et al.*; Jacobsen *et al.* 1990; Kurosaki *et al.* 1988, 1990; Molano *et al.* 1979; Ride and Barber

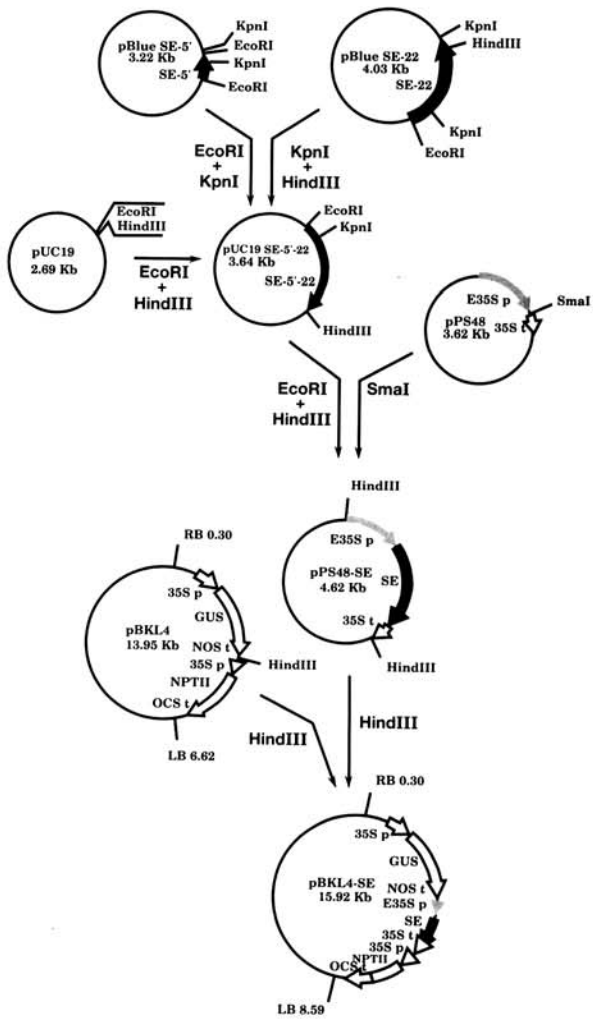

Fig. 9. Construction of the plant transformation vector pBKL4-SE harboring the SE2 gene regulated by an enhanced 35S promoter and the 35S terminator. See text for an explanation of the components of the various vectors.

1990; Roby and Esquerre-Tugaye 1987; Vad *et al.* 1991). Two acidic class III chitinases from cucumber and chickpea have also been analyzed for reaction products, using thin-layer chromatography (TLC), and were both characterized as endochitinases (Metraux *et al.* 1988; Vogelsang and Barz 1993). Likewise, the pattern of reaction products detected after limited hydrolytic action of the SE2 enzyme on chitin is typical of endochitinases. However, SE2 also releases appreciable amounts of monomer even after short incubation times (10% after 1 hr), and is capable of hydrolyzing chito-oligomers, including the dimer, into the monomer during prolonged action (Table 1). Whether this observed additional exochitinase activity is a unique feature of SE2, or merely an effect of assay conditions and differences in methods of analysis, remains to be investigated. However, recent TLC analysis has disclosed a similar additional exochitinase activity for the basic class III barley chitinase 6 (K. M. Kragh, unpublished). Thus, we expect that this ability to completely hydrolyze chitin and chito-oligomers to release *N*-acetylglucosamine monomer may turn out to be a more general character of plant chitinases.

Recently, two acidic proteins, D2 and D3, with apparent MW of 28 kDa, were found to accumulate in the intercellular fluid of sugar beet leaves after infection with tobacco necrosis virus (TNV) or treatment with salicylic acid (SA) (Fleming *et al.* 1991; Fleming 1992). The antibody raised against SE2 reacted strongly with D2 and D3, and a 12 residue long N-terminal amino acid sequence of D3 was shown to be identical to that of SE2. This strongly suggests that the extracellular proteins D2 and D3, inducible by virus or chemicals, are chitinase isozymes identical or closely related to SE1 and SE2 induced by fungal infection. This also suggests that SE2, like D2 and D3, is excreted into the intercellular space. In accordance with this, SE2 protein was found in the intercellular fluid of transgenic tobacco plants. Furthermore, immunocytochemical studies on transsections of *Cercospora*-infected sugar beet leaves only revealed antigens to the chitinase 4 antibody in the intercellular space, whereas no intracellular reaction was found (J. Nielsen, personal communication). Together, this demonstrates that SE2, like the other class III chitinases that have been analyzed with respect to subcellular localization (Lawton *et al.* 1992, Metraux *et al.* 1989, Vogel-sang and Barz 1993), is extracellularly localized.

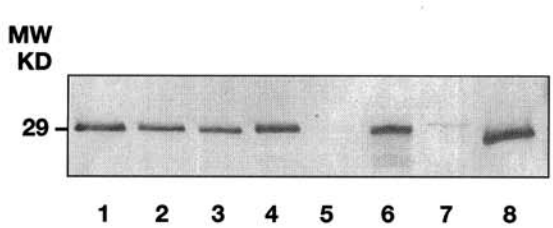

Fig. 10. Expression of SE2 protein in transgenic tobacco plants (*N. benthamiana*). Leaf homogenates and intercellular washing fluid (IWF) were fractionated by SDS-PAGE and probed with antibody against chitinase 4. Lane 1, purified SE2 (100 ng). Lanes 2–4, leaf homogenate, three different primary transformants transformed with pBKL-SE (5 μg of total protein). Lane 5, control, transformed with pBKL4– (5 μg). Lane 6, IWF pool from mature leaves of 10 transformants (5 μg). Lane 7, cell homogenate (pool) after removal of IWF (5 μg). Lane 8, purified chitinase 4 (100 ng).

Although the amino acid sequence of SE2 only shows 10% identity with that of the class IV chitinase 4, the two proteins show serological relationship when analyzed under denaturing conditions. Comparing the primary structure of the two chitinases reveals that no consecutive sequence identities are shared that may form a potential common epitope. Such an epitope generally consists of at least six amino acids (Scheidtmann 1989). Whether the recognition of SE by the chitinase 4 antibody, and vice versa, is caused by a common tertiary conformation is not known. A similar serological relationship between two structurally unrelated chitinases has previously been reported from bean (Margis-Pinheiro *et al.* 1991). Interestingly, these two chitinases also represent classes III and IV chitinases.

The potential involvement of SE2 in the resistance response against invading pathogens may be reflected by the differences in the mRNA induction between susceptible and tolerant plants in relation to the infection cycle of *C. beticola*. The fungus penetrates the leaf within 3–4 days of inoculation and approximately 5–6 days later the first symptoms are observed as necrotic spots. Therefore, the strong induction of chitinase mRNA in the susceptible cultivar from day 12, at a time where the fungus is well established in the leaf, is probably too late to have any significant effect. In the tolerant cultivar, where the symptoms appear 1 or 2 days later and are less severe, the late induction of SE2 mRNA is somewhat weaker. In these plants, on the other hand, a strong transient "burst" of SE2 mRNA is observed at day 8 or 9, which may be part of a more rapid defense response being responsible for the delayed and moderated disease development. To our knowledge, this marked additional transient chitinase mRNA expression has not been reported from other plant-pathogen interactions.

Whether the acidic chitinase SE2 has a main function as a direct antifungal agent against chitin-containing phytopathogenic fungi is not known. In the preliminary infection trial, the transgenic tobacco plants overexpressing SE2 chitinase showed no difference in the time of appearance or severity of symptoms compared to control plants. However, as the number of plants was limited and the plant material very heterogeneous, larger infection trials using F_1-generation plants have to be performed in order to evaluate the full antifungal potential of SE2 against *Cercospora* and other fungi. In addition, transgenic tobacco plants expressing double and triple combinations of SE2, a basic chitinase and a basic β-1,3-glucanase are being produced. Succeeding infection experiments may unveil the applicability of these proteins in the efforts to develop plants with increased fungal disease resistance.

MATERIALS AND METHODS

Biological materials.

For large-scale purification of chitinase enzymes, leaves of *Beta vulgaris* L. 'Monova' (Maribo Seed), were collected in the field at Maribo Italia, Bologna, Italy. The plant material was heavily infected with *C. beticola*. For induction experiments and mRNA isolation, seeds of *B. vul-*

garis 'Monova' (*Cercospora*-susceptible) and '+Tol' (tolerant), were sown in clay mixed peat (Pindstrup no.2, Pindstrup Mosebrug A/S, Denmark) and placed in a growth chamber with 11-hr day at 25/18° C (day/night) and 70% RH. The plants were supplied with water containing 0.1% (v/v) fertilizer (Hornum, Broeste A/S, Denmark) twice a day. *C. beticola* (isolate FC573, supplied by Earl G. Ruppel, USDA, Fort Collins, CO), and *C. nicotianae* (Ell & Ev.) (supplied by Margaret E. Daub, Dept. of Plant Pathology, North Carolina State University) were cultured on potato-dextrose agar (Difco Laboratories, Detroit, MI) at room temperature. For spore production, the fungi were grown on V8-juice agar at 13° C and 24 hr cool-white light.

Inoculation.

Six-week-old sugar beet plants were inoculated with a conidia suspension of *C. beticola*, adjusted to 1.2×10^4 spores per milliliter in 0.05% (w/v) Tween 20, by spraying the adaxial leaf surface to saturation. Control plants were mock-inoculated with 0.05% Tween 20. The plants were placed in a "mist chamber" at 30° C, 100% RH, and 24 hr cool-white light for 5 days, followed by incubation at 30° C, 80% RH, and 24 hr light. At specified times after inoculation, leaves were harvested, frozen in liquid nitrogen, and stored at −80° C until use. Plants of *N. benthamiana* were inoculated with a spore suspension of *C. nicotianae,* containing 5×10^3 spores per milliliter, and treated as described above.

Purification of the acidic chitinase.

The initial steps in the purification of the acidic chitinase (SE), namely homogenization, heat treatment, and ammonium sulphate precipitation, were performed as described by Vad *et al.* (1991). After dialysis against 10 mM Tris-HCl, pH 8.0, containing 1 mM DTT and 1 mM BAM, SE was purified on two successive FF Sepharose Q anion-exchange columns (Pharmacia LKB, Uppsala, Sweden), equilibrated in the Tris buffer. Bound proteins, including SE, were eluted with salt; from the first column in one step with 0.5 M NaCl and from the second column with a linear gradient from 0 to 0.5 M NaCl in 20× column volume. Fractions containing chitinase activity, as assayed by the radioactive enzyme assay described below, were pooled and dialyzed against 25 mM Bis-Tris, pH 7.0. Further purification of the chitinase was achieved on a Polybuffer Exchanger 74 chromatofocusing column (Pharmacia LKB) equilibrated in the same Bis-Tris buffer. The column was eluted with Polybuffer 74, diluted 1:10, and adjusted to pH 3.6 with iminodiacetic acid. This created a linear pH gradient from pH 7.0 to 3.6. At the final pH, SE was still retained on the column and could be eluted by addition of 0.3 M NaCl to the Polybuffer 74. SE was finally purified by anion-exchange fast protein liquid chromatography (FPLC) on a Mono P HR 5/20 column (Pharmacia LKB) equlibrated in 25 mM Bis-Tris, pH 7.0. Proteins were eluted with a linear salt gradient from 0 to 0.3 M NaCl in 30 ml of the Bis-Tris buffer.

Polyacrylamide gel electrophoresis and immunoblotting.

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using tricine SDS-PAGE (Schagger and von Jagow 1987) minigels (16.5 %) on the Mighty Small system (Hoefer, CA) or a Phast System with precast 10–15% acrylamide gels (Phast Gel gradient 10-15; Pharmacia LKB) and low molecular weight standards from Pharmacia LKB. Determination of isoelectric point (pI) was performed on the Phast System (Pharmacia LKB) using Phast Gels IEF 3-9 and IEF standards pH 3-10 (Pharmacia LKB). Running and silver staining of the gels was performed according to the manufacturer's instructions. Proteins separated by SDS-PAGE were electroblotted onto a 0.45-μm nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using a semidry blotting system (JKA-Biotech, Copenhagen, Denmark) (Kyhse-Andersen 1984). The membrane was incubated overnight with polyclonal rabbit antibodies raised (Dakopatts A/S, Copenhagen, Denmark) against specific sugar beet chitinase isozymes, diluted 1:500 (Mikkelsen *et al.* 1992). Using an alkaline phosphatase-conjugated secondary antibody (pig anti-rabbit IgG; Dakopatts) the blot was developed by staining with nitrobluetetrazolium (Kyhse-Andersen 1984).

Hydrolytic activities, reaction products, and affinity to chitin.

Chitinase activity was determined by the radiochemical assay described by Molano *et al.*(1977) using the modifications described by Kragh *et al.* (1990). SE was appraised for potential lysozyme activity using the assay described by Selsted *et al.* (1980).

For the analysis of chitinase reaction products, [^{3}H]-labeled chitin (200 μg ~ 7.2×10^5 cpm) was incubated with 10 μg of SE2 enzyme at 40° C for 1 or 6 hr in 300 μl of 10 mM ammonium acetate, pH 5.0. After centrifugation (5 min at 20,000 *g*), the supernatant, containing the soluble hydrolysis products, was removed, frozen at −80° C and freeze-dried. To determine the reaction products after prolonged hydrolysis, [^{3}H]-chitin was incubated with 10 μg of enzyme for 24 hr. After centrifugation, the supernatant was removed and incubated with a further 5 μg of enzyme for 12 hr. HPLC analysis of the reaction products was performed using a modification of the system described by Usui *et al.* (1990) on a silica column (A-003-10, 4.6 × 250 mm; YMC-Pack, Japan). Elution was effected with water/acetonitrile (71.5:28.5, v/v). The flow rate was 0.8 ml/min, and the UV absorbance was monitored at 210 nm. From 4–19 min after sample injection fractions of 267 μl were collected and the radioactivity was measured by scintillation counting. As standards, 15 μg of *N*-acetylglucosamine (Sigma), di-*N*-acetyl-chitobiose, tri-*N*-acetyl-chitotriose, and tetra-*N*-acetyl-chitotetraose (Biocarb, Sweden) were used.

Regenerated chitin was prepared as described by Molano *et al.* (1977) and modified by Kragh *et al.* (1990). The ability of purified SE to bind to chitin was investigated at varying pH. The buffers used were: 20 mM citric acid (pH 2.5), 20 mM acetate (pH 4.0), 10 mM Bis-Tris (pH 6.0), and 10 mM Tris (pH 8.0 or 10.0). All buffers contained 1 mM DTT. After loading a sample of purified SE2 and incubation for 30 min at 4° C, the column was washed with the starting buffer and subsequently eluted with salt

(1 M NaCl) or a buffer at lower pH.

Protein concentration was determined according to Bradford (1976) using the Bio-Rad protein assay kit (Richmond, CA) with γ-globulin as standard.

Tryptic digestion and separation of tryptic peptides.

The purified SE2 protein was transferred to 20 mM NH_4HCO_3 on a NAP-5 column and freeze-dried. One hundred micrograms of protein was redissolved in 200 microliters of 0.2 M Tris-HCl, pH 8.4, containing 7 M guanidine hydrochloride and carboxymethylated as described previously (Kragh *et al.* 1991). Digestion of the protein with trypsin, separation of the tryptic peptides, and amino acid sequencing was performed as detailed by Kragh *et al.* (1991).

Synthesis of an SE PCR probe.

For the synthesis of a polymerase chain reaction (PCR) probe for SE, mRNA was isolated from sugar beet leaves of cv. Monova, 14 days after inoculation with *C. beticola*, using the Dynabeads Oligo(dT)25 mRNA purification kit (Dynal A/S, Oslo, Norway) according to the manufacturer's instructions. Synthesis of first-strand cDNA and two successive PCR amplifications were performed in a DNA Thermal Cycler (Perkin Elmer Cetus, Norwalk, CT) using the protocol from the manufacturer with the following modifications. First-strand cDNA synthesis was extended to 45 min and the PCR cycles were as follows: one cycle at 94° C for 1 min, 37° C for 2 min, 50° C for 1 min, and 72° C for 40 min; five cycles at 94° C for 1 min, 37° C for 2 min, and 72° C for 10 min; and 30 cycles at 94° C for 1 min, 42° C for 2 min, and 72° C for 5 min, followed by a final extension at 72° C for 20 min. After the second PCR amplification, the product was digested with *Bam*HI and *Hin*dIII and cloned into the pUC19 plasmid (Boeringer Mannheim, Mannheim, Germany). The sequence was determined essentially according to the dideoxynucleotide-chain termination method of Sanger *et al.* (1977) using the Sequenase kit (U.S. Biochemicals, Cleveland, OH).

Screening of the sugar beet cDNA library.

Total RNA for cDNA library construction was isolated from sugar beet leaves, cv. Monova, 12 days after inoculation with *C. beticola* and poly $(A)^+$ RNA was isolated by oligo(dT) cellulose chromatography (Chirgwin *et al.* 1979). cDNA was synthesized by Stratagene (La Jolla, CA) and ligated into the *Eco*RI site of λ-ZAP II. cDNA clones encoding SE were identified by plating 1×10^6 plaques from the λ-ZAP II library and screening duplicate filters (Hybond-N; Amersham Int., Buckinghamshire, England) with the insert from the PCR clone, ^{32}P-labeled by the random priming method using the "Prime It" kit from Stratagene. Hybridization was performed at 67° C in 2× SSC, 0.1% SDS, 10× Denhardt's solution, and 50 μg/ml denaturated salmon sperm DNA. The filters were washed twice in 2× SSC, 0.1% SDS and twice in 1× SSC, 0.1% SDS for 15 min at 67° C. The positive clones were subjected to *in vivo* excision according to Stratagene's protocol.

Northern and Southern blotting and hybridization.

Total RNA for Northern blotting experiments was extracted at different time points after inoculation with *C. beticola* essentially according to the method of Collinge *et al.* (1987). Absorbance measurements (260 nm) and agarose gel electrophoresis were performed to estimate RNA concentrations and to test that the RNA was intact. Formaldehyde electrophoresis was performed essentially as described by Maniatis *et al.* (1982) followed by blotting onto a Hybond-N membrane. A ^{32}P-labeled probe was prepared from a 1,070-bp SE cDNA clone by random priming (Stratagene). Northern blots were prehybridized for 2 hr at 68° C in 4× SSC, 0,5% SDS, 4 × Denhardt, 1 mM EDTA, 200 μg/ml denatured salmon sperm DNA, and 1 μg/ml poly A. The SE probe and 7% dextran sulphate was added to the prehybridization solution before hybridization for 20 hr at 68° C. The membrane was washed successively for 2 × 30 min at 42° C in 2 × SSC, 0.1% SDS, and 0.1 × SSC, 0.1% SDS. To establish that equal amounts of RNA had been loaded in each lane, the membrane was subsequently probed with an unidentified 300-bp sugar beet cDNA clone (Con), hybridizing to a mRNA exhibiting constitutive expression in all sugar beet leaf tissue.

Genomic DNA was isolated from sugar beet leaves, cvs. Monova and +Tol, according to Dellaporte *et al.* (1983). Aliquots of 20 μg of DNA were digested with selected restriction enzymes, fractionated by electrophoresis on a 0.8% agarose gel and transferred to a Hybond-N membrane. Hybridization with the SE cDNA probe was performed at 65° C overnight following the protocol from Amersham. A high-stringency membrane wash was performed as follows: 2 × 15 min at 65° C in 2 × SSC, 0.1% SDS followed by 1 × SSC, 0.1% SDS and 0.1 × SSC, 0.1% SDS.

Generation of transgenic tobacco plants.

A culture of *Agrobacterium tumefaciens*, strain LBA 4404 (Ooms *et al.* 1982), was transformed with the plant transformation vector pBKL4 harboring a full-length SE gene (pBKL4-SE) flanked by a NPTII and a GUS gene using a freeze/thaw method (An *et al.* 1988). The pBKL4 vector is a modification of pBI121 (Clontec Lab., Inc., Palo Alto, CA) in which the mutated NPTII gene has been replaced with a nonmutated NPTII (J. Brunstedt, unpublished). Before introduction into pBKL4, the SE chitinase gene was supplied with an enhanced 35S promoter and a 35S terminator by subcloning into pPS48. pPS48 is a derivative of pCAMVCN (Pharmacia LKB) in which the 35S promoter, CAT gene, and NOS terminator have been replaced with a polylinker between an enhanced 35S promoter and a 35S terminator (P. Stougaard, unpublished).

Agrobacterium-mediated transformation of leaf disks from *N. benthamiana* was performed essentially as described by Horsch *et al.* (1985). Transformed shoots were regenerated on selective MS medium (Murashige-Skoog) containing kanamycin (300 mg/L) and carbenicillin (800 mg/L). Expression of the β-glucuronidase reporter gene was tested in leaf tips of green shoots using the GUS assay

described by Jefferson *et al.* (1987). The expression and subcellular localization of the SE2 protein in leaves of primary transformants showing high GUS activity was analyzed by Western blotting of leaf homogenates and intercellular washing fluids (IWF). IWF was isolated according to Fleming *et al.* (1991).

Primary transformants were transplanted into soil. When the regenerated plants had 8–10 mature leaves they were inoculated with *C. nicotianae*. Transgenic plants without the SE2 gene were used as control.

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