

Primary structure and expression of acidic (class II) chitinase in potato

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Abstract

Infection of potato (*Solanum tuberosum*) leaves by the late blight fungus *Phytophthora infestans* or treatment with fungal elicitor leads to a strong increase in chitinase activity. We isolated cDNAs encoding acidic (class II) chitinases (ChtA) from potato leaves and determined their structures and expression patterns in healthy and stressed plants. From the total number of cDNAs and the complexity of genomic DNA blots we conclude that acidic chitinase in potato is encoded by a gene family which is considerably smaller than that encoding basic (class I) chitinase (ChtB). The deduced amino acid sequences show 78 to 96% identity to class II chitinases from related plant species (tomato, tobacco) whereas the identity to basic chitinases of potato is in the range of 60%. RNA blot analysis revealed that both acidic and basic chitinases were strongly induced by infection or elicitor treatment and that the induction occurred both locally at the site of infection and systemically in upper uninfected leaves. In contrast, a differential response to other types of stress was observed. Acidic chitinase mRNA was strongly induced by salicylic acid, whereas basic chitinase mRNA was induced by ethylene or wounding. In healthy, untreated plants, acidic chitinase mRNA accumulated also in an organ-, cell-type- and development-specific manner as revealed by RNA blot analysis and *in situ* RNA hybridization. Relatively high transcript levels were observed in old leaves and young internodes as well as in vascular tissue and cells constituting the stomatal complex in leaves and petioles. Lower, but appreciable mRNA levels were also detectable in roots and various flower organs, particularly in sepals and stamens. The possible implications of these findings in pathogen defense, development and growth processes are discussed.

Introduction

Plants respond to pathogen attack by the activation of a large variety of defense mechanisms which can be classified into different categories based on their temporal and spatial patterns of expression [22]. Typical examples include the hypersensitive response (HR), the synthesis of antimicrobial phytoalexins, the deposition of lignin, phenolic compounds, callose and proteins into the cell wall, and the accumulation of numerous defense-related proteins. Among these are the

pathogenesis-related (PR) proteins which were originally identified in tobacco after infection by tobacco mosaic virus (TMV), but subsequently also detected in a large variety of plant species. Thus PR proteins are now assumed to be ubiquitous in the plant kingdom [13, 23, 28, 54]. As a result of extensive structural and functional information and the discovery of novel PR proteins, their classification has been extended from 5 to 10 families, including, among proteins with unknown function, well-characterized enzymes such as chitinases (families 3 and 8) and 1,3- β -glucanases (family 2) [58].

Chitinases are unique amongst the plant defense proteins in that their major substrate *in vitro*, β -1,4-

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession numbers U49969 (ChtA2) and U49970 (ChtA1).

linked *N*-acetylglucosamine (chitin), does not exist in plants but constitutes one of the major structural cell-wall components of many fungi [3]. Since chitinase activity in plants drastically increases after infection it is assumed that chitinases are involved in protecting plants against fungal pathogens [5]. In support of such a function, it could be demonstrated that purified chitinases are not only capable of degrading isolated fungal cell walls but to also inhibit *in vitro* growth of chitin-containing fungi, particularly when tested in combination with 1,3- β -glucanases [1, 7, 34, 51]. In addition, expression of chitinase genes in transgenic plants resulted in enhanced resistance to certain fungal pathogens [7, 30], particularly when coexpressed with 1,3- β -glucanase genes or other defense-related genes [18, 57, 67]. However, contrary data also exist [38, 41]. Furthermore, in tobacco the phenomenon of systemic acquired resistance (SAR) has clearly been correlated with the systemic expression of at least 9 families of genes, including those encoding acidic (class II) chitinase and 1,3- β -glucanase [6, 62]. Those encoding the basic isoforms are not activated which is surprising in view of their considerably higher antifungal activity [51].

All plants analyzed to date contain multiple forms of chitinase which have been divided into several different classes on the basis of their structural and functional properties [11, 23]. Class I chitinases are usually basic proteins localized in the vacuole. They contain a glycine-rich N-terminal domain with chitin-binding properties which is connected to the catalytic domain via a proline-rich hinge region, and a short C-terminal domain providing the information for targeting to the vacuole. Class II chitinases share about 60% sequence identity with class I chitinases, but they are usually acidic, extracellular proteins which lack the chitin-binding domain and the C-terminal targeting signal. Class III chitinases are also extracellular proteins but lack sequence similarity to the class I and class II enzymes and were therefore grouped into a separate family of PR proteins [58]. Class IV chitinases resemble class I chitinases in having a cysteine-rich domain, a hinge region and a related catalytic domain, but due to a few gaps they are smaller proteins. Class V chitinases are represented by two proteins from tobacco with rather low chitinase activity that are unrelated to other plant chitinases but share sequence similarity to bacterial exochitinases [35]. The majority of chitinases characterized to date, including members of all classes described above, are induced by infection or other types of stress, but they are also expressed in an

organ-, cell-type- and development-specific manner in uninfected plants which suggests that these enzymes have an additional as yet unrecognized function in plant growth and development. This is further supported by the identification of a few specific chitinases that are constitutively expressed and restricted to specific organs or cell types, such as flowers or epidermal cells [20, 63].

In potato, infection with *Phytophthora infestans* or treatment with an elicitor from the same fungus, has been shown to lead to the accumulation of several PR proteins among which chitinases and 1,3- β -glucanases have been identified [21]. Potato chitinases have further been demonstrated to constitute a heterogeneous family of proteins from which members of class I and class II have been purified and characterized [64]. In addition, cDNA sequences, genome organization and expression patterns of class I chitinases have previously been reported, suggesting that increased enzyme activity upon infection or other stress treatments as well as the organ-, cell-type- and development-specific expression is primarily regulated at the level of mRNA abundance [4, 20]. In the present paper we report the molecular cloning, sequence and expression analysis of acidic (class II) chitinase from potato and relate it to class I chitinase in order to increase our understanding of the function of this divergent PR protein superclass during local and systemic resistance as well as its potential involvement in plant growth and development.

Materials and methods

Plant material and stress treatments

Potato plants (*Solanum tuberosum* L. cvs. Datura and Désirée) were grown from tubers in a greenhouse under controlled conditions for 6 to 8 weeks [21] unless otherwise stated. The first to sixth fully expanded leaves (8–15 cm in length) were used for experiments. All stress treatments, such as inoculation with *P. infestans*, treatment with elicitor, sodium salicylate or ethylene, and wounding, were performed as previously described with detached leaves which were placed with their petioles into vials containing water or the indicated solutions [4, 21, 50].

Isolation and nucleotide sequence analysis of chitinase cDNAs

A cDNA library in the bacteriophage λ NM1149, that was constructed from RNA of potato (cv. Datura) leaves that had been treated with elicitor for 24 h [4], was screened with a tobacco cDNA probe encoding acidic chitinase (Tach1 [29]; kindly provided by Dr H.J.M. Linthorst, Department of Chemistry, Leiden University, Leiden, Netherlands). Out of 180,000 recombinant pages plated, 72 clones were found to hybridize with the probe at moderate-stringency conditions ($2\times$ SSC, 60 °C), 20 of which were selected for further purification. After four rounds of plaque purification the longest cDNAs were subcloned into pUC9 and sequenced by the dideoxynucleotide chain-termination method [8] using T7 DNA polymerase (Pharmacia, Freiburg, Germany). Suitable sequencing primers were synthesized on an Applied Biosystems Model 380A DNA synthesizer (Applied Biosystems, Foster City, USA). Sequences were analyzed on a VAX computer (Digital Equipment, München, Germany) using the software developed by the Genetics Computer Group (Madison, WI) [15].

RNA and DNA isolation and blot hybridization

Total RNA was isolated as described by Lois *et al.* [31], denatured, separated on 1.2% agarose-formaldehyde gels, transferred to nylon membrane (Hybond-N, Amersham-Buchler, Braunschweig, Germany) according to the conditions recommended by the manufacturer and cross-linked with UV light. Labelling of DNA probes and hybridization of the blots were performed as described [50], except that the temperature was increased to 60–65 °C, as formamide was not included in the hybridization solution. Blots were washed three times in $0.2\times$ SSC at 60 °C. In some experiments, washing conditions of low stringency ($2\times$ SSC, 50 °C) were used, followed by washing steps of successively higher stringency.

In situ hybridization

Leaf tissue was fixed and embedded in paraffin according to the procedure described by Schmelzer *et al.* [49]. Two series of consecutive sections were prepared for hybridization to sense (control) and antisense RNA probes which were generated from linearized plasmids with T3 or T7 polymerase and uridine 5'-[α -³⁵S]thiotriphosphate (400 Ci/mmol; Amersham-

Buchler, Braunschweig, Germany) according to previously described procedures [50], with the exception that the pronase treatment was extended (500 μ g/ml for 12 min). The conditions for *in situ* hybridization, washing and microautoradiography were the same as previously described [49, 50] except that slides were exposed to NTB-2 autoradiographic emulsion at 4 °C for 3 weeks.

Results

Isolation and sequence analysis cDNAs for class II chitinases

In potato leaves, chitinase and 1,3- β -glucanase activities increase strongly and coordinately upon infection with *P. infestans* or treatment with an elicitor preparation from the fungal culture filtrate [21, 50]. It has also been demonstrated that the mRNA amounts of the basic isoforms of both enzymes strongly increase after both treatments [4]. To isolate additional cDNAs, a library complementary to mRNA of elicitor-treated potato leaves was screened with the tobacco cDNA, Tach1, which encodes the acidic, extracellular chitinase PR-P [29]. Out of 180,000 recombinant potato clones, 72 were found to hybridize strongly with the tobacco probe; 20 of these were purified and analyzed.

The sequences of two clones, each representing several cDNAs that varied in length, are shown in Figure 1. While ChtA2 is apparently full-length, ChtA1 is truncated at the 5' end. Both sequences are identical at 95.5% of the positions, and only 3 out of 11 nucleotide differences in the coding region also result in an amino acid exchange. A few additional cDNAs differed from ChtA2 in only few base pairs (97.3–99.8% identity) giving rise to the same deduced amino acid sequence (not shown). A putative polyadenylation signal (AATAAA) is found at position 861 in ChtA2 and at the same relative position in all other cDNAs. Additional authentic and variant (AAGAAA) polyadenylation signals are found in those clones which have an extended 3'-untranslated region (not shown). Comparison of ChtA1 and ChtA2 to sequences in the database revealed high similarity to acidic (class II) chitinases from other plants, such as tomato [9, 14]), petunia [29]), and tobacco [29, 43]), ranging from 93.2% to 73.7% identity. The similarity to basic (class I) chitinases of potato and other plants is considerably lower ($\leq$ 60% identity).

ChtA1		
ChtA2	CAGAGGAAAAATGAAGTTCAATGTATCACCAGTTGCACTCTCTGTCTCTTTTCTTGT	60
	M K F N V S P V A L S C L F F L F	17
ChtA1	T	
ChtA2	CCTAACAGAACTTTAGCACAAATGCTGGTTCAATTGTAACGAGGAATTATTCGAACA	120
	L T E T L A Q N A G S I V T R E L F E Q	37
	▲	
ChtA1		
ChtA2	AATGTTGAGTTT TAGGAACAATGATGATGTCTCGGCAAAGGATTCTACACTTATGATGC	180
	M L S F R N N D V C P G K G F Y T Y D A	57
ChtA1	C	
ChtA2	ATTTCATAGCTGCAGCTAACTCGTTTCCAGCTTTTGGTACTACTGGTGATGATACCGCCCG	240
	F I A A A N S F P A F G T T G D D T A R	77
ChtA1		
ChtA2	TAAGAAGGAAATGGCTGCCTTTTTCGGTCAAACATCTCAGGAACTAATGGTGGTAGTGC	300
	K K E M A A F F G Q T S H E T N G G S A	97
ChtA1		
ChtA2	AGGAACATTTACTGGAGGATATTGCTTTGTTAGGCAAATAGATCAGTCAGAAAGATACTA	360
	G T F T G G Y C F V R Q I D Q S E R Y Y	117
ChtA1	T	
ChtA2	TGGCAGAGGACCTATCCAATTGACACACCAATCTAACTACGAACGAGCTGGACAAGSTAT	420
	G R G P I Q L T H Q S N Y E R A G Q G I	137
ChtA1	T	
ChtA2	TGGTGTGGACAAGACCTAGTGAACAACCTGATTAGTAGCCACAGATCCTATAATATC	480
	G V G Q D L V N N P D L V A T D P I I S	157
	▲	
ChtA1		
ChtA2	ATTCAAAACAGCAATATGGTTCTGGATGACAGCACAGGATAATAAGCCATCATGCCACAA	540
	F K T A I W F W M T A Q D N K P S C H N	177
ChtA1	C T	
ChtA2	CGTTATCATTTGGCAATGGACGCCATCCGCCGAGATACGGCAGCTAATAGAGTGCCAGG	600
	V I I G Q W T P S A A D T A A N R V P G	197
	P	
ChtA1	T	
ChtA2	GTACGGTGTGATTACCAATATCATTAACGGTGGACTTGAATGCGGGATGGGTCCGAATAC	660
	Y G V I T N I I N G G L E C G M G P N T	217
	Q	
ChtA1		
ChtA2	TGCAGTGGAAGTCGAATTGGATTTTACAGGAGGTATTGTGGTATGCTGAATGTTCCAC	720
	A V E S R I G F Y R R Y C G M L N V P T	237
ChtA1	T	
ChtA2	TGGTGAAAACCTTGGACTGTAACAATCAAAGGAACTTCGCCAGGGCTAAGCGTCTGTTACA	780
	G E N L D C N N Q R N F A Q G *	252
ChtA1	GTAC	
ChtA2	TATATAGAATGCAAAATTATGTTAT...GTATTACTATTACTCGTGGAGATTGTTTCAGTC	836
ChtA1		
ChtA2	TATAAGTTATATTGGATGTAATGAATAAAGGATTCTGTATGCCCATTTAGAATAGGGA	847
	▼(A16)	896
ChtA2	GATATATTTGCTCTTTAAAAATTAAAGG	925

Figure 1. Nucleotide sequences of cDNA clones encoding acidic chitinases of potato and predicted amino acid sequences of the products. For clone ChtA1 only nucleotides that differ from ChtA2 are given and differences in the predicted amino acid sequences are highlighted in bold letters. Gaps and missing nucleotides are indicated by dots (.). The putative cleavage site of the N-terminal signal peptide is marked by an arrow, the start and stop codons and the putative polyadenylation signal are underlined, and the attachment site of the poly(A) tail of 16 nucleotides (A16) in ChtA1 is indicated by an triangle (▼).

The deduced amino acid sequence of ChtA2 is presented in Figure 2 and compared with two other chitinases from potato: SK2, an abundant basic protein of the stylar transmitting tract [63], and ChtB3, one of the pathogen-inducible, basic (class I) chitinases of leaves [4]. In all three chitinases hydrophobic signal peptides can be identified, the cleavage of which in

ChtA2 according to the rules of von Heijne [61] would give rise to a mature protein of 229 amino acids with a calculated M_r of 24 917 and a pI of 5.73. The properties of the mature ChtA1 protein are virtually identical (M_r 24 947, pI 5.73) and both are in fair agreement with those determined experimentally for an extracellular chitinase (M_r 29 000, pI 4.2 and 4.4) purified

ChtA2	MKPNVSPVALSCLFPLFTETLAQNAGS.....	28
ChtSK2	...MRLVLVLAWFSVLCCLKVLAQDVGS.....	24
ChtB3	EFTIPSLFLSLLLNLSAEEQCGSQAGGALCAPGLCCSKFGWCGNT	45
ChtA2	IVTRELFQMLSRNNNDVCPGK..GF	52
ChtSK2	LINKNLFERIFLHRNDAACAAK..GF	48
ChtB3	NDYCGPGNCOSQCPGPGPGSGDLGGVTSNSMFDQMLNHRNDNAOQGNF	95
ChtA2	YTYDAFIAAANSFPAGTTGDDTARKKEMAAFFGQTSHETNGSAGTFTG	102
ChtSK2	YTYEAFITATKSEAFGTTGDTNTRKKEIAAPLAQTSHETGGNATAPDG	98
ChtB3	YSYNAFISAAGSFPFGTTGDTTARKREIAAPLAQTSHETGGWPSAPDG	145
ChtA2	GYCFV.....RQIDQSERYGRGPIQLTHQSNYERA	133
ChtSK2	PYSWGYCFKQEQSGPGDYCVASQQWPCAPGKYFGRGPTQISYNYNYPGA	148
ChtB3	PYAWGYCFLRBQSGPGDYCTPSSQWPCAPGRKYFGRGPIQLTHQSNYGP	195
ChtA2	GQIGVGCQDLVNNDPLVATDPIISFKTAIWFMTAQDNKPSCHNVITIGW	183
ChtSK2	GRAIGV...NLLNNPDLVANDAVVSFKTALWFMTAQDNKPSAHVDITGRW	196
ChtB3	GRAIGV...DLLNNPDLVATDSVISFKSAIWFMTAQDNKPSCHDVITGRW	243
ChtA2	TPSAADTAANRVPGYVITNIINGGLECGMGPTAVESRIGFYRRYCGML	233
ChtSK2	SPSAADSAGRVPGFVITNIINGGLECGSGNMQMDNRIGFYRRYCGIL	346
ChtB3	QPSGADQAANRVPGFVITNIINGGLECGHGSDSRVQDRIGFYRRYCGIL	293
ChtA2	NVPTGENLDCNNQRNFAQG.....	252
ChtSK2	GVDPGNNLDCANQRPFQ.....	264
ChtB3	GVSPGDNLDCNQRSPFGNGLVDTV	318

Figure 2. Comparison of amino acid sequences of different potato chitinases. The amino acid sequence predicted for acidic chitinase ChtA2 (cv. Datura) was aligned with the predicted sequences of chitinase SK2 (cv. Roxy) which is specifically expressed in pistils [63] and the basic (class I) chitinase ChtB3 (cv. Datura) which is induced in leaves by pathogen infection [4]. Gaps (.) were introduced for optimal alignment. Amino acids common to any two of the sequences are shaded. The putative cleavage sites of the N-terminal signal peptide which is present in all proteins and of the C-terminal extension in ChtB3 are marked by arrows. The chitin-binding or hevein domain located at the N-terminus of mature ChtB3 is underlined, as are the conserved amino acids of the putative active site, and the tyrosine residue identified to be essential for activity in maize chitinase [59] is marked by a bold dot (●).

from potato leaves [64]. The absence of C-terminal vacuolar targeting signals (Figure 2) is in accordance with the extracellular localization of both ChtA2 and SK2 [63, 64]. This and the absence of chitin-binding domains and proline-rich hinge regions in ChtA2 and SK2 clearly identifies both proteins as class II chitinases although their amino acid sequences are identical at only 56.7% of the positions. Between ChtA2 and ChtB3 60% of the amino acid positions are identical.

Despite this overall sequence divergence amongst the different types of potato chitinases, highly conserved regions can be identified which include, at least in part, the putative active site (Figure 2). The tyrosine residue which was found to be essential for enzymatic activity in chitinase of *Zea mays* is conserved in the majority of basic chitinases sequenced to date [59]. Interestingly, in all class II chitinases, except SK2, this tyrosine is exchanged for another amino acid, mostly

for serine (Ser-128) as in potato chitinase ChtA2 (Figure 2). At present it is unknown whether or not this has any functional implications.

The amino acid sequence of ChtA2 shares the highest similarity with acidic (class II) chitinases from other plants, ranging from 93.3% to 95.6% identity for Chi3 and pcht28 from cultivated and wild tomato species, respectively [9, 14], and 77.6% to 78.4% for PR-P and PR-Q from tobacco [29, 43]. In contrast, no significant similarity is found between the ChtA2 protein and class III chitinases from cucumber and tobacco [26, 36], and only a low degree of amino acid sequence identity (40.5%) is shared with class IV chitinase of bean [33].

Induction of acidic and basic chitinase mRNAs by various stimuli

The induced expression of acidic chitinase genes in potato leaves in response to different kinds of stress was studied by RNA blot hybridization and compared to that of basic chitinases (ChtB) genes. The latter and genes encoding basic 1,3- β -glucanase (GluB) have previously been shown to be strongly and coordinately activated by infection, elicitor treatment, ethylene treatment or wounding [4]. In healthy, untreated leaves mRNA encoding ChtA is present at very low levels (Figure 3). Inoculation of potato leaves with *P. infestans* or treatment with an elicitor preparation from the fungal culture medium leads to a strong mRNA accumulation which is similar for both types of chitinase. In general, induction by elicitor treatment occurs somewhat faster than by infection, with significant increases being detectable at 6 h and 12–18 h, respectively, but finally infection results in accumulation of higher mRNA levels (Figure 3, [4]).

Treatment of potato leaves with salicylic acid (SA) or ethylene resulted in a differential response of acidic and basic chitinases: ChtA mRNA was rapidly induced by SA, with maximum levels being reached as early as 12 h after the onset of treatment, whereas ChtB mRNA levels were not affected. In contrast, ethylene treatment gave rise to high levels of ChtB mRNA levels at 24–48 h with no or only little effect on ChtA mRNA levels. The optimum SA concentration for the induction of ChtA mRNA was in the range of 0.4–0.8 mM, but significant induction was also obtained with 0.1 mM whereas a concentration of 1.0 mM was already suboptimal (Figure 4). Induction by wounding was similar to that observed after ethylene treatment, however, the mRNA levels were generally lower (Fig-

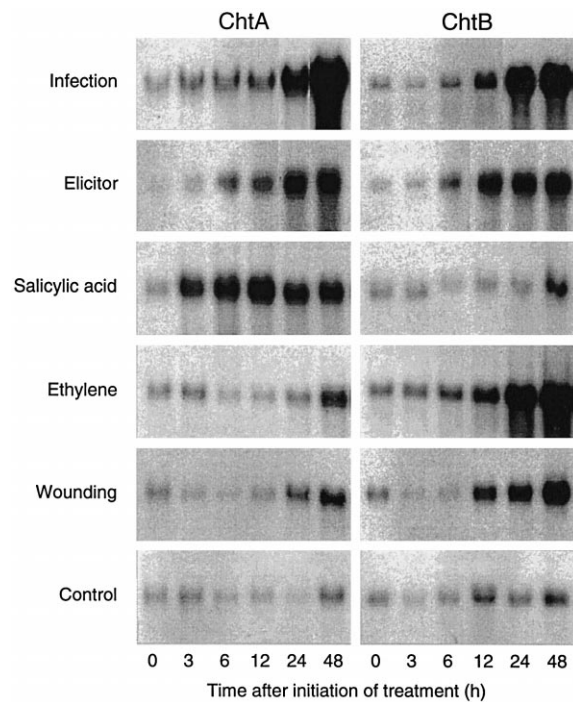


Figure 3. Accumulation of acidic and basic chitinase mRNAs in response to different types of stress. Detached potato leaves (cv. Datura) were infected with *Phytophthora infestans*, treated with elicitor (100 $\mu\text{g}/\text{ml}$), sodium salicylate (1 mM), ethylene, or wounded and incubated for the times indicated. Control leaves were maintained in parallel under otherwise identical conditions. Total extractable RNA (10 $\mu\text{g}/\text{lane}$) was separated on agarose gels, blotted onto nylon membranes and hybridized with radiolabelled probes for acidic (ChtA) and basic (ChtB) chitinase which detected mRNAs of 1.2 kb and 1.3 kb in size, respectively.

ures 3 and 5). In control leaves, sometimes a low and variable increase in mRNA levels was observed which is, in part, attributed to a wound response, since all experiments were performed with detached leaves.

A direct comparison of the relative mRNA levels of acidic and basic chitinases after infection, SA or ethylene treatment, or after wounding is presented in Figure 5, where it is also compared to the mRNA levels of acidic and basic 1,3- β -glucanases (GluA and GluB). Obviously, ChtA is the most strongly induced mRNA species after infection with *P. infestans* reaching a level after 96 h which is comparable to that induced after 12 h of SA treatment. The mRNAs encoding basic chitinase or basic 1,3- β -glucanase were most strongly induced after 12 h of ethylene treatment and to a lesser extent by the other stress treatments whereas GluA mRNA amounts increased to similar levels by 12 h of SA or ethylene treatment (Figure 5). For compar-

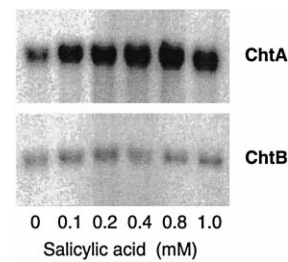


Figure 4. Induction of acidic and basic chitinase mRNAs in potato leaves by different concentrations of salicylic acid. Detached leaves were placed for 24 h with their petiole into vials containing sodium salicylate solutions of the indicated concentrations. RNA blots (10 $\mu\text{g}/\text{lane}$) were hybridized with radiolabelled probes for acidic (ChtA) and basic (ChtB) chitinase.

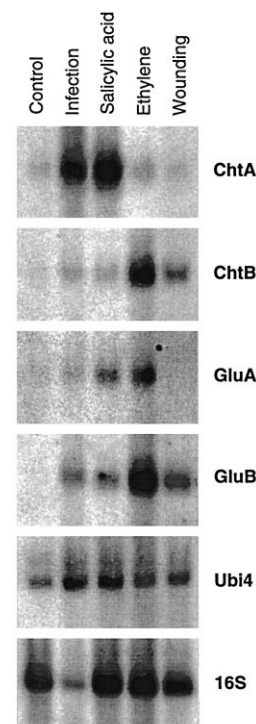


Figure 5. Comparison of accumulated mRNA amounts in potato leaves after different kinds of stress. Total RNA was extracted from detached leaves after infection with *Phytophthora infestans* (96 h), treatment with 1 mM sodium salicylate (12 h), ethylene (12 h), wounding (12 h) or untreated controls. RNA blots (10 μg RNA per lane) were hybridized with radiolabelled probes for acidic (ChtA) and basic (ChtB) chitinase of potato, acidic (GluA) and basic (GluB) 1,3- β -glucanase of potato, polyubiquitin (ubi4) of parsley [19] and 16S ribosomal RNA (16S) from maize [24].

ison, identical RNA blots were also hybridized with probes encoding polyubiquitin (ubi4) of parsley [19] and 16S ribosomal RNA (16S) of maize [24]. Ubi4 mRNA levels significantly increased by all applied

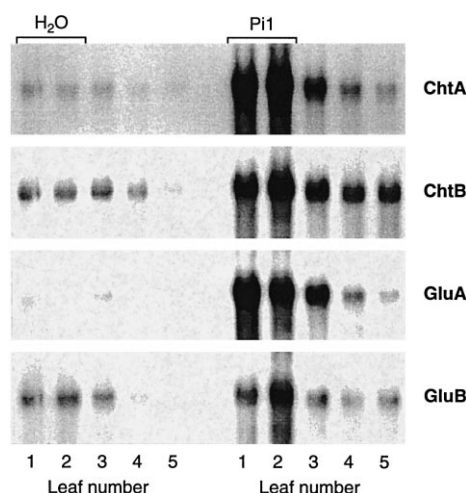


Figure 6. Local and systemic induction of chitinase and 1,3- β -glucanase mRNAs in potato leaves following infection by *Phytophthora infestans*. Two basal leaves of six-week-old potato plants (cv. Datura) were inoculated with spores of *P. infestans*, race 1 (Pi1; compatible interaction) or treated with water. After 4 days of incubation, RNA was isolated from the infected leaves (1–2) as well as from the uninfected, acropetal leaves (3–5) of the same plant. RNA blots (10 μ g RNA per lane) were hybridized with radiolabelled probes for acidic (ChtA) and basic (ChtB) chitinase, and acidic (GluA) and basic (GluB) 1,3- β -glucanase of potato.

stress treatments, in contrast to the constitutive expression reported for cultured parsley cells [19, 53]. 16S rRNA levels were more or less constant in all samples except for a decrease that was observed after infection. This decrease is presumably the result of specific RNA degradation since the amount of loaded total RNA was nearly identical in all samples as demonstrated by ethidium bromide staining of the gel (not shown).

In general, the results shown here are consistent with those reported earlier for the induction of basic chitinase in potato leaves [4]. They are also consistent with expression studies in tobacco, in which a preferential induction of acidic and basic chitinase by SA and ethylene, respectively, has been reported [6, 62].

Since in tobacco a strong correlation of the phenomenon of systemic acquired resistance (SAR) and systemic expression of different PR proteins, in particular acidic chitinase and 1,3- β -glucanase was observed [6, 62], we also studied systemic gene expression utilizing the available potato probes. Following inoculation of two lower leaves of a potato plant with *P. infestans*, a strong local accumulation of mRNAs encoding acidic and basic chitinases and 1,3- β -glucanases can be observed in the directly infected tissue, but in addition, increased levels of all four types of transcripts,

i.e. acid and basic chitinases and 1,3- β -glucanases can also be detected in upper uninfected leaves of the same plant (Figure 6). This result is in accordance with our previous observation of systemic expression of basic 1,3- β -glucanase [50] but it differs from similar studies in tobacco in which only the acidic isoforms were systemically induced [6, 62]. However, in potato it has not yet been established that systemic gene activation after infection with *P. infestans* correlates with the SAR phenotype.

Development-, organ- and cell-type-specific accumulation of acidic chitinase mRNA

Expression of acidic chitinase genes in different organs of healthy, untreated potato plants was analyzed by RNA blot hybridization. In young leaves, harvested from the top of 12-week-old plants, a low level of ChtA mRNA was detectable whereas old leaves, harvested from the bottom of the plants, contained significantly higher transcript levels (Figure 7). When the internodes of the same plants were analyzed, an opposite gradient of ChtA mRNA amounts was observed, with higher mRNA levels being present in young compared to old stems. Roots also contained only low amounts of ChtA mRNA, as did the separated organs of fully developed flowers with highest levels being present in sepals, stamens and carpels and lowest in petals (Figure 7).

Cell-type-specific expression of ChtA genes was studied by *in situ* RNA hybridization. In these experiments paraffin-embedded serial sections of infected and uninfected leaves were hybridized with ^{35} S-labelled antisense and sense (control) RNA probes generated by *in vitro* RNA transcription of cDNA in the pBS vector with T3 or T7 RNA polymerase [50]. Infection sites were identified by immunohistochemical localization of fungal material using polyclonal antiserum raised against *Phytophthora* cell walls [50] and by identification of necrotized tissue areas. In the vicinity of infection sites a strong accumulation of ChtA mRNA could be detected (Figure 8A) whereas little or no signal was observed in the necrotic or collapsed cells of the infection site proper. In sections taken from the same leaf at a distance from infection sites the detectable levels of ChtA mRNA were considerably lower (Figure 8B), however, in these sections, as well as in those taken from uninfected leaves, ChtA mRNA could clearly be localized in cells of the vascular system and in cells identical with or in close proximity to guard cells (Figures 8B and C). In leaf sections which included the mid vein, the vascular cells con-

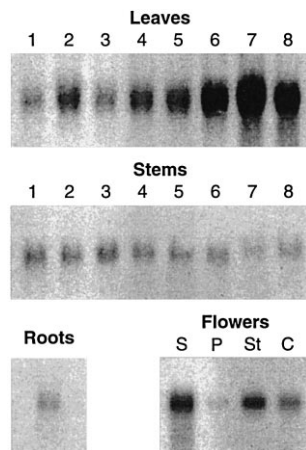


Figure 7. Acidic chitinase mRNA levels in different organs of potato. Leaves of a 10- to 12-week-old plant were divided into 8 groups of two consecutive leaves each: 1, youngest leaves (top of the plant); 2–7, intermediate stages; 8, oldest leaves (bottom of the plant). The internodes of the same plant were grouped accordingly. From the roots one sample was taken and fully developed flowers were separated into sepals (S), petals (P), stamens (St) and carpels (C). Total extractable RNA (10 μ g/lane) was separated, blotted and hybridized with the radiolabelled probe ChtA2.

taining high levels of ChtA mRNA could be assigned to the phloem (Figure 8D). In leaf sections with a sufficient quality of tissue preservation and in cross-sections through the petiole (Figure 8E) the labelling within the stomatal complex could clearly be assigned to the guard cells. Control treatment of tissue sections with sense RNA gave no specific labelling (Figure 8F).

Discussion

The comparison of different cDNAs encoding acidic chitinase in potato revealed only few differences in their nucleotide sequences. In the coding regions most of the nucleotide differences are silent resulting in proteins which are either identical or different in only 3 amino acid positions when the most divergent cDNAs are compared. Whether this difference is due to allelic polymorphism of a single gene in the tetraploid potato genome or whether different genes are present and expressed could not be decided on the basis of the available sequences. But the limited sequence divergence and number of hybridizing bands on DNA blots suggests that acidic chitinase in potato is encoded by a rather small gene family of presumably not more than two members per haploid genome. In contrast,

the gene family encoding basic chitinase in potato is of considerable complexity [4].

The proteins encoded by ChtA cDNAs show all the characteristic features of class II chitinases, including a high degree of similarity to related proteins of other plants, particularly tomato [9, 14] and tobacco [29, 43], the absence of the chitin-binding or hevein domain and the lack of the C-terminal extension which in tobacco was shown to be necessary and sufficient for targeting basic chitinases to the vacuole [39]. In addition, the predicted properties (M_r , pI, extracellular localization) are also in fair agreement with those experimentally determined for a chitinase that was purified from the intercellular washing fluid of potato leaves [64].

Comparing the induction of acidic and basic chitinase mRNAs in potato leaves revealed similarities as well as differences. Both types of mRNA accumulate in response to different kinds of stress, including infection, treatment with elicitor, SA, ethylene, or wounding. However, a direct comparison revealed considerable differences in the relative mRNA amounts, in particularly after ethylene or salicylic acid treatment (Figures 4 and 5). ChtA gene expression is strongly induced by SA and only weakly by ethylene or wounding, whereas the opposite is true for ChtB gene expression. A similar difference, although less pronounced, was observed for acidic and basic 1,3- β -glucanases (Figure 5). In tobacco, the same kind of differential expression of acidic and basic PR proteins has been reported and, more importantly, a correlation between induction by SA of acidic PR proteins and of the SAR phenotype has been established [6, 62]. From these observations two conclusions have been drawn, (1) acidic PR proteins are involved in establishing resistance whereas the basic ones are not, and (2) ethylene is unlikely to be part of the signaling chain leading to defense gene activation specific for establishing SAR. From our results it appears that in potato the same general mechanisms of regulating PR protein gene expression are utilized, but a correlation between PR protein gene expression and induced resistance remains to be established. The systemic activation following infection of both acidic and basic chitinase and 1,3- β -glucanase gene expression in potato leaves (Figure 6) is in contrast to the preferential systemic induction of only acidic PR proteins as observed in tobacco [6, 62].

Although systemic resistance can be induced in potato by local infection with *P. infestans*, treatment with fungal cell-wall components or arachidonic acid, a correlation with systemic activation of defense responses or systemic SA accumulation has not been

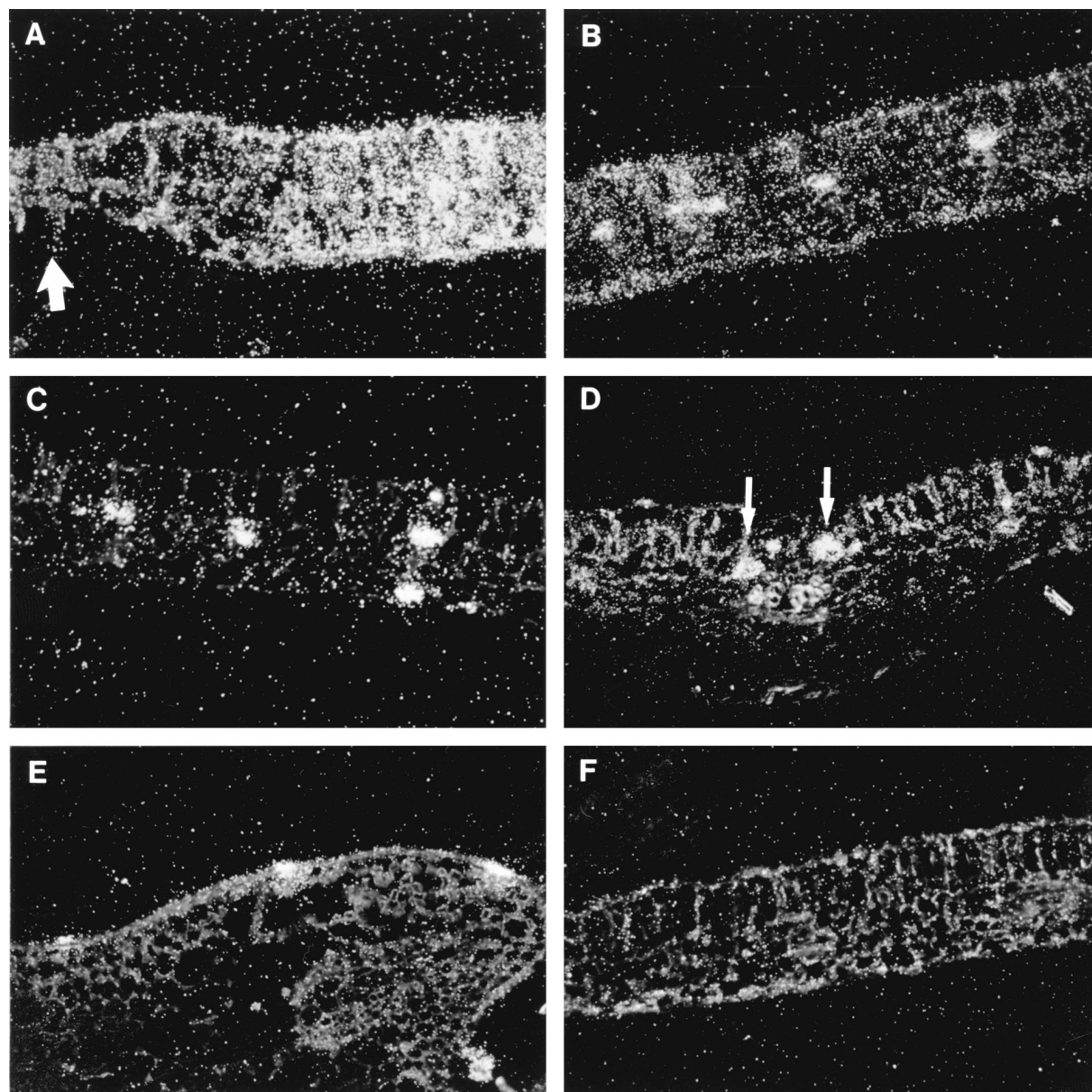


Figure 8. *In situ* localization of acidic chitinase mRNA in cross-sections of different potato tissues (cv. Désirée) by hybridization with ^{35}S -labelled ChtA2 antisense probes. A. Young potato leaves were inoculated with *Phytophthora infestans*, race 1 (compatible interaction) for 48 h. The arrow points to the center of an infection site. B. Section of the same leaf as A but taken at a distance from the infection site. C. Section of an uninfected control leaf. D. Section through the center of an uninfected control leaf including the mid-vein. Arrows point to labelled cells of the phloem. E. Section through a petiole with intense labelling of guard cells. F. Section through an uninfected leaf after hybridization with labelled sense RNA (as control for unspecific binding of probe).

observed [10, 12, 16, 55], and it has not been possible to induce resistance in potato by treatment with SA [12] or any other chemical activator of resistance that is active in other plants. However, in contrast to constant systemic SA levels, a local increase occurred

rapidly in response to infection of potato leaves concomitant with a local but not systemic induction of PR protein 1 [12]. Other (defense) responses reported to be activated by SA treatment include acidic chitinase (see above), osmotin [66], and catalase [40]. Thus, in

potato SA seems to be involved in mediating local resistance and local defense gene activation the same way as in other systems, however, in contrast to other systems, a systemic translocation and signaling function during the establishment of SAR is not obvious. But also in tobacco, cucumber and *Arabidopsis* in which the induction of SAR is tightly associated with the production of SA [32, 37, 56] compelling genetic and biochemical evidence suggests that SA is not likely to be the translocated signal itself but rather an indispensable signaling component exerting its function downstream of another long distance signal [44, 47, 60].

In addition to induction by infection or other kinds of stress, expression of many PR proteins is controlled in a development-, organ- and cell-type-specific manner in healthy plants. High levels of chitinase and 1,3- β -glucanase enzyme activities and mRNA amounts are generally found in old or senescing leaves, as well as in roots, whereas they are low or absent in young leaves [4, 27, 52]. Hence it is not surprising to find a similar pattern of ChtA mRNA accumulation in potato, with higher transcript levels being present in old as compared to young leaves (Figure 7). This is contrasted by an opposite gradient of mRNA accumulation in internodes in which higher transcript levels are associated with young in comparison to old tissue. Roots also contained an appreciable amount of ChtA mRNA albeit lower than that found for basic chitinase and 1,3- β -glucanase [4]. Although gene expression is clearly regulated during development, the function of acidic chitinase in this process remains obscure.

High levels of chitinase and 1,3- β -glucanase are usually found in specific organs or cell types of flowers. In a few cases it has been demonstrated that there are distinct isoforms, structurally different from those induced in leaves, that are highly expressed in stigma, style and ovaries. Examples are chitinase SK2 and 1,3- β -glucanase sp41, which have been demonstrated to be abundant stilar matrix proteins in potato and tobacco, respectively [42, 63]. In most cases, however, the structures of chitinases or 1,3- β -glucanases that were detected in flowers have not been elucidated, leaving the possibility open that isoforms are being detected that are structurally different from the probes utilized for detection. The same is of course true for ChtA. Nonetheless, it is clear from our results that potato flowers, in addition to containing transcripts encoding chitinase ChtB and SK2 that were previously localized in carpels [4, 63], also contain significant amounts of mRNA identical or similar to ChtA. The function of these diverse chitinases in flowers is unclear, but it is

reasonable to assume that it is a protective one. Particularly the wet surface of pistils potentially presents a highly susceptible tissue for infection by fungal or bacterial pathogens and high, constitutive levels of different defense proteins may be aimed at protecting potential offspring against pathogen ingress and to assure efficient reproduction. However, in the absence of additional functional data a direct participation of chitinases in the pollination and/or reproduction process, such as trimming or modification of polysaccharides containing *N*-acetylglucosamine, cannot be excluded.

Participation in the defense program is the suggested prime function of chitinase in leaves. The accumulation of ChtA mRNA in potato in response to infection by *P. infestans* at the site of fungal ingress as shown by *in situ* RNA hybridization further supports such a role. The pattern of mRNA accumulation is consistent with the strong increase in chitinase activity previously reported [21, 50] and it occurs in coordination with an increase in abundance of mRNA encoding basic chitinase which is also initiated at infection sites [4, 17]. This also suggests that the increase in enzyme activity is transcriptionally regulated and at least partially due to increased mRNA abundance.

The localization of ChtA mRNA in vascular tissue (phloem) and in guard cells suggests specific functions of the gene product in these cells. Again, this function might be related to defense against pathogens although no experimental evidence for this assumption is available. Stomata are natural openings in leaves which are the preferred, essential or exclusive site of entry into the leaf for many pathogens, particularly downy mildews, rust fungi and bacterial pathogens. Therefore, acidic chitinase may function as a preformed defense barrier at this vulnerable location. An alternative or additional function of high, constitutive chitinase levels near guard cells could be the generation of signal molecules from invading pathogens thereby activating other defense responses in the surrounding tissue. In support of this hypothesis it has been demonstrated in some instances that chitinases are capable of releasing from fungal pathogens *N*-acetyl-glucosamine oligomers which are active elicitors [2, 25, 45, 46]. Interestingly, specific expression of a reporter gene driven by a class III chitinase promoter in guard cells of *Arabidopsis* has been reported [48]. The specific accumulation of ChtA mRNA in or near vascular tissue as demonstrated for potato is also not without precedent and it has been observed in *Arabidopsis* and in tomato after infection with *Cladosporium fulvum* [48,

65]. High levels of ChtA in this tissue could be directed against vascular pathogens and/or be the consequence of higher SA levels in the phloem as they occur in tobacco and cucumber leaves upon infection [32, 37].

In conclusion, genes encoding acidic chitinase in potato display complex patterns of expression which in addition to induction by pathogen infection and other kinds of stress is obviously controlled by endogenous factors in a development-, organ- and cell-type-specific manner. It will be interesting to determine whether these patterns are the result of differential expression of specific members of the ChtA gene family and/or whether different regions or elements within their promoters are responsible for the observed differential expression. We have isolated genes encoding ChtA and are currently studying their structures and expression with the aim to identify a suitable molecular marker for SAR induction and to further elucidate their function in the resistance response of potato.

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