



STRUCTURE AND FUNCTION OF CHITIN-BINDING PROTEINS

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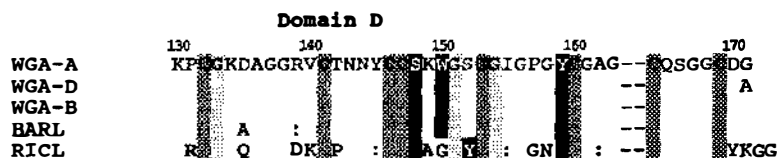
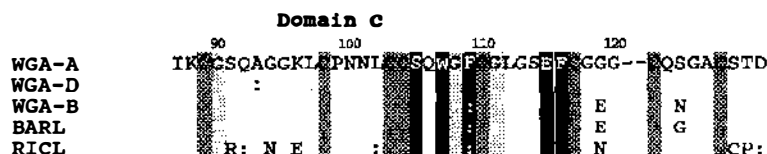
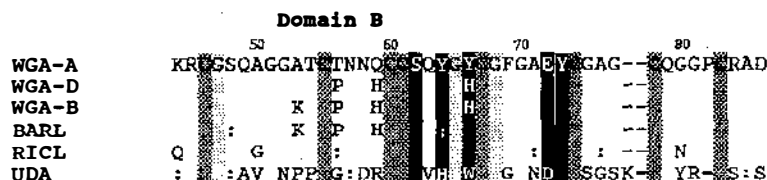
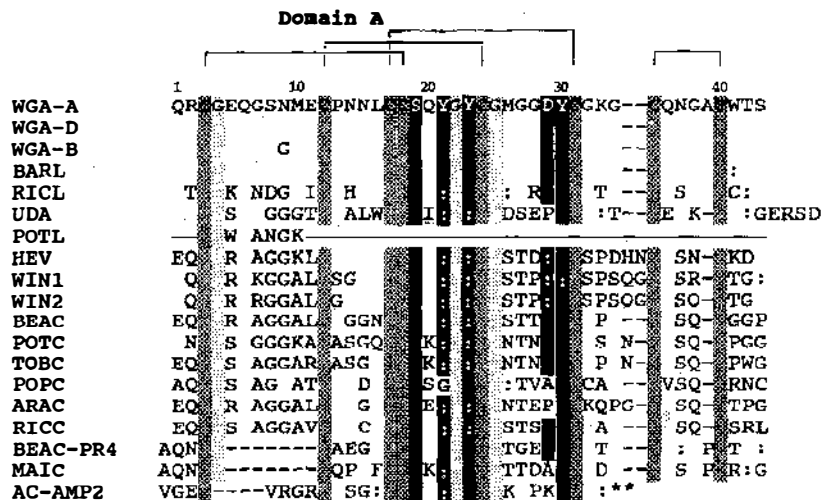
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CONTENTS

INTRODUCTION	592
PROTEIN STRUCTURE AND PROCESSING	593
<i>Gramineae Lectins</i>	593
<i>Amaranth Antimicrobial Peptides</i>	595
<i>Hevein and Wound-Induced Proteins</i>	597
<i>Class I Chitinases</i>	598
<i>Stinging Nettle Lectin</i>	599
<i>Solanaceae Lectins</i>	599
SPATIAL EXPRESSION AND GENE REGULATION.....	600
<i>Gramineae Lectins</i>	600
<i>Hevein and Wound-Induced Proteins</i>	601
<i>Class I Chitinases</i>	602
<i>Stinging Nettle Lectin</i>	603
<i>Solanaceae Lectins</i>	603
BIOLOGICAL ACTIVITIES	604
<i>Antifungal Activity</i>	604
<i>Insect Antinutrient Activity</i>	606
EVOLUTIONARY CONSIDERATIONS	607
CONCLUSIONS.....	608

INTRODUCTION

Plants synthesize a wide array of proteins capable of reversibly binding to affinity matrices composed of chitin, a β -1,4-linked biopolymer of *N*-acetylglucosamine (GlcNAc). All chitin-binding proteins for which the amino acid sequence is known contain a common structural motif of 30–43 amino acids with several cysteines and glycines at conserved positions (Figure 1). This polypeptide motif will henceforth be referred to as the chitin-binding



domain. Although the term “chitin-binding proteins” denotes the family of proteins containing one or more chitin-binding domains, it must be emphasized that the binding affinity of these proteins is not restricted to chitin, but may extend to various complex glycoconjugates containing GlcNAc or *N*-acetyl-D-neuraminic acid (NeuNAc) as building blocks. Since the natural ligand of chitin-binding proteins is not known with certainty, we can only speculate about their exact physiological role in the plant. Chitin, the most obvious natural ligand, has never been detected in higher plants. Instead, it is an important structural component of the cell wall of fungi and of the exoskeleton of many invertebrates, such as insects and nematodes. This fact, along with other circumstantial evidence discussed below, points to a role in host defense, although other functions are not excluded (32). In this review we discuss the structural relationships between chitin-binding proteins, their posttranslational processing, their localization in plant tissues, their induction under stress conditions, and their antibiotic activities against microorganisms and insects. Emphasis is placed on chitin-binding proteins with lectin (or agglutinin) properties, i.e. proteins capable of cross-linking GlcNAc- or NeuNAc-containing polymers due to the presence of multiple chitin-binding domains.

PROTEIN STRUCTURE AND PROCESSING

Gramineae Lectins

The best characterized chitin-binding protein is wheat germ agglutinin (WGA), the lectin from wheat. In hexaploid wheat (*Triticum aestivum*) WGA exists in three variant forms (isoelectins A, B, and D) encoded by their respec-

Figure 1 Aligned amino acid sequences of the chitin-binding domains of several chitin-binding proteins. The one-domain proteins are aligned with domain A of the four-domain protein WGA (wheat germ agglutinin), and the two-domain protein *Urtica dioica* agglutinin (UDA) is aligned with domains A and B of WGA. Only those residues that differ from the sequence top line are shown, and sequence identity is indicated by blanks. Conservative substitutions are denoted with two dots (substitutions among the amino acid groups: A, S, and T; N and Q; D and E; I, L, M, and V; H, K, and R; F, W, and Y). Gaps introduced for optimal alignment are represented by dashes. Disulfide linkages shown at the top line of domain A are identical in other domains of WGA (135). Conserved cysteines and glycines are shown in dark grey and light grey boxes, respectively. Residues identical or homologous to the residues of WGA involved in saccharide binding are shown in black boxes. The thin line represents amino acids that have not been determined. The end of mature *Amaranthus caudatus* (Ac-AMP2) protein is denoted with asterisks. Abbreviations and references: WGA-A, WGA-D, WGA-B, wheat germ agglutinin isoelectins (100, 112); BARL, barley lectin (55); RICL, rice lectin (132); UDA *Urtica dioica* agglutinin or stinging nettle lectin (8); POTL, tryptic peptide from potato lectin (16); HEV, hevein (126); WIN1, WIN2, wound-induced proteins from potato (113); BEAC, bean basic chitinase (25); POTC, potato basic chitinase (41); TOBC, tobacco basic chitinase (109); POPC, poplar wound-induced chitinase (WIN8; 85); ARAC, *Arabidopsis thaliana* basic chitinase (106); RICC, rice basic chitinase (143); BEAC-PR4, bean acidic PR (pathogenesis-related) 4 chitinase (64); MAIC, maize seed chitinase A (46); Ac-AMP2, *Amaranthus caudatus* antimicrobial peptide (19).

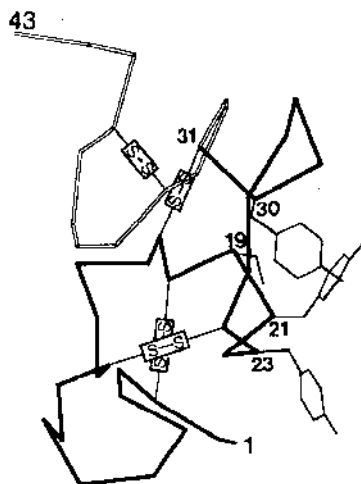


Figure 2 Stereo view of the α -carbon backbone of the 43-residue structural domain of WGA. Each corner represents a $C\alpha$ position. The four disulfide bridges are designated S-S. The core region (residues 3–31) is outlined in black and includes the four side chains (at positions 19, 21, 23, and 30) implicated in sugar binding. The spatially distinct C-terminal linker region (residues 31–43) is represented as a double line. (Reproduced with permission from C. S. Wright.)

tive diploid genomes (93, 137). All three WGA isolectins consist of two identical 171-residue polypeptides that associate to form 36-kd dimers. The WGA polypeptides show remarkable redundancy in their structure; they are composed of four repetitive domains of 42–43 amino acids (137; Figure 1). Crystal structure analysis revealed that the four independent domains are folded similarly and stabilized by four identically positioned intradomain disulfide bridges (133, 134). Three of the four disulfides are nested and form the well conserved inner core region (domain positions 3–31), whereas the ends have a higher degree of structural flexibility and sequence variability (Figure 2). The two polypeptide chains of the WGA dimer associate in a head-to-tail fashion. Although it was previously thought that dimerization was required for the formation of functional saccharide-binding pockets (134), recent high-resolution X-ray diffraction results of cocrystals of a WGA-ligand complex have raised the possibility that saccharide binding can occur entirely within a single domain (139). Additional binding strength, however, comes from the cooperative interaction between the two opposing domains that form the binding site, each of which is located on a different monomer (136). Amino acid residues involved in saccharide binding are the triad of aromatic residues at domain positions 21, 23, and 30, a serine at domain position 19, and a glutamate or aspartate at domain position 29 (Figure 2). The latter provides a hydrogen bond with the *N*-acetylated secondary amine function of the sugar.

Lectins similar to WGA according to immunological, biochemical, and carbohydrate-binding criteria are also present in other Gramineae, such as rye (*Secale cereale*; 94) and barley (*Hordeum vulgare*; 94), rice (*Oryza sativa*; 120), couch grass (*Agropyrum repens*; 30), and false brome grass (*Brachypodium sylvaticum*; 92). These lectins are all 36-kD dimers composed of two identical 18-kD subunits (Figure 3). In cultivated rice species the majority of the 18-kD subunits undergo proteolytic cleavage that yields two subunits of 8-kDa and 10-kDa held together by intramolecular disulfide bridges (118; Figure 3). Chitin-binding lectins with cysteine/glycine-rich amino acid profiles similar to WGA have also been found in representatives of the Papaveraceae (the greater celandine, *Chelidonium majus*; 91) and the Phytolaccaceae (poke-weed, *Phytolacca americana*; 128). The amino acid sequences of these lectins have not been determined.

The cDNAs that encode the WGA isolectins A,B, and D (100, 112), barley lectin (55), and rice lectin (132) have been sequenced. Gramineae lectins are highly homologous at both the amino acid and the nucleotide levels (for a review see 96). The Gramineae lectin cDNA clones encode proteins with three distinct portions (Figure 3): (a) an N-terminal putative signal sequence (26–28 amino acids), (b) a sequence with four repetitive domains corresponding to the mature lectin polypeptide (171 amino acids for the wheat and barley lectins, 173 amino acids for the rice lectin), and (c) a C-terminal propeptide that contains a high mannose glycan that is not present in the mature lectin (15 amino acids for the wheat and barley lectins, 26 amino acids for the rice lectin). The signal sequence has been shown to be cotranslationally removed (62). The C-terminal propeptide first undergoes cotranslational glycosylation and the propeptide is then cleaved during transport or upon arrival in the vacuoles (131). Studies of barley preprolectin expression in transgenic tobacco plants have revealed that inactivation of the glycosylation site does not affect folding, processing, or targeting of the mature lectin (131), whereas deletion of the entire propeptide results in missorting and secretion (7). When fused to an otherwise secreted protein, such as cucumber chitinase, the barley lectin propeptide sequence can potentiate its redirection to the vacuole (6). These experiments indicate that the barley lectin propeptide is both necessary and sufficient for vacuolar targeting.

Amaranth Antimicrobial Peptides

The smallest and structurally most simple chitin-binding proteins are the antimicrobial peptides, Ac-AMP1 and Ac-AMP2, which are found in seeds of amaranth (*Amaranthus caudatus*; 19). Ac-AMP1 and Ac-AMP2 are 29 and 30 amino acids in length, respectively, and have identical sequences except for an additional arginine residue at the C-terminus of Ac-AMP2. The Ac-AMP polypeptides probably associate to form dimers. Alignment of the Ac-AMP

sequences with the chitin-binding domain of WGA reveals identical residues at approximately 50% of the positions after insertion of a 4-residue gap in the

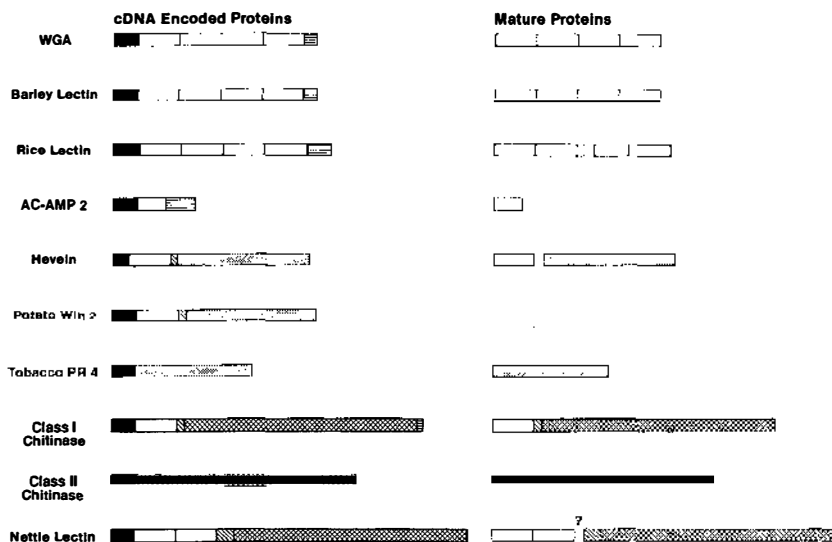


Figure 3 Primary structure of the chitin-binding family and related proteins. The preproteins of Gramineae lectins (55, 100, 112, 132) consist of a signal sequence (solid box), four chitin-binding domains (open boxes), and a C-terminal extension propeptide (CTPP) domain (horizontal stripe box) containing an *N*-linked high-mannose glycan. The glycosylated CTPP is cleaved to yield the mature 18-kDa subunits of the dimeric lectins. The mature rice lectin undergoes another posttranslational cleavage to yield polypeptides of 8 and 10 kDa.

The deduced amino acid sequence of Ac-AMP2 cDNA contains a signal sequence, a mature Ac-AMP2 domain, and a CTPP domain possessing a putative *N*-glycosylation site (W. F. Broekaert, unpublished). It appears that proAc-AMP2 is posttranslationally processed to form mature Ac-AMP2.

Hevein cDNA encodes a signal sequence, a chitin-binding domain (mature hevein), a hinge region (diagonal box), and a C-terminal domain (stippled box; 18, 126). The prohevein is posttranslationally cleaved to yield a mature hevein and a C-terminal polypeptide (53). The deduced amino acid sequence of potato *win* (wound-induced) genes (113) shows a high degree of homology to both domains of prohevein; however, nothing is known about posttranslational modifications of these proteins. The deduced amino acid sequence of tobacco PR4 cDNA consists of a signal sequence and a polypeptide homologous to the C-terminal domain of hevein (38, 57).

The deduced amino acid sequence of class I chitinases (25, 41, 85, 109) shares a similar structure: signal sequence, a chitin-binding domain, a variable hinge region, and a chitinase domain (cross-hatched box). The open reading frame of class II chitinases (58, 86) contains a signal sequence and a chitinase domain.

Stinging nettle lectin cDNA encodes a signal peptide, two chitin-binding domains (UDA), a hinge region, and a chitinase domain (56). Prostinging nettle lectin is probably posttranslationally processed to yield two cleavage products of mature UDA and a chitinase domain polypeptide.

Ac-AMP sequence (Figure 1). Since the Ac-AMPs are shorter than the canonic 43-residue chitin-binding domain and contain three instead of four disulfide bridges, they appear to be a truncated variant of the chitin-binding domain. In fact, the Ac-AMP sequences correspond to the "inner conserved core region" of the consensus chitin-binding domain as defined by Wright et al (139). It is also noteworthy that all residues implicated in saccharide binding by the WGA domains are conserved in the Ac-AMPs (Figure 1). The fact that Ac-AMPs have been demonstrated to bind to chitin-affinity matrices supports the current view of Wright et al (136, 139) that a single "inner core domain" is responsible for ligand binding. Unlike WGA, however, the Ac-AMPs do not agglutinate red blood cells; this indicates that a single chitin-binding domain is not sufficient for carbohydrate cross-linking activity.

The Ac-AMP2 cDNA encodes an 86-amino-acid protein containing an *N*-terminal putative signal sequence (25 amino acids), a domain corresponding to mature Ac-AMP2, and a 31-amino-acid C-terminal extension (W. F. Broekaert, unpublished). Although there is no sequence similarity between the latter domain and the C-terminal propeptide of Gramineae lectins, both domains have common structural features, such as the occurrence of an *N*-glycosylation site, the presence of a valine residue 8 amino acids ahead of the glycosylation site, and the relative abundance of amino acids with short hydrophobic side chains (alanine and valine). These properties are also shared by the propeptide of the basic β -1,3-glucanase of tobacco (111, 115).

Hevein and Wound-Induced Proteins

A protein structurally homologous to a full-size single repetitive domain of WGA is a major protein in the latex of rubber tree (*Hevea brasiliensis*; 126). This small 5-kDa monomeric protein, called hevein, is 43 amino acids in length and has identical residues at 40–53% of the positions relative to the WGA domains (Figure 1). The conserved residues include all eight cysteines, three glycines, and the residues of the carbohydrate-binding site at positions 19, 21, 23, 29, and 30 (139). Like the Ac-AMPs, hevein binds to chitin but does not agglutinate erythrocytes (124). The fact that hevein is a monomer and binds to chitin is consistent with the recent finding that dimerization is not required for interaction with the saccharide ligand (136).

The cDNA that encodes hevein is much larger than the nucleotide sequence necessary to encode a single chitin-binding domain protein. The hevein cDNA encoded protein is a 204-amino-acid preproprotein consisting of a putative signal sequence, the mature hevein domain, and a hinge region followed by a C-terminal domain (Figure 3; 18). A study of the biosynthesis of preprohevein both in vitro and in rubber tree latex has revealed that the signal sequence is cotranslationally removed and that the resulting prohevein is incompletely cleaved in a subsequent posttranslational processing step. Because of this

incomplete processing, rubber tree latex contains mature hevein, a 15-kDa protein corresponding to the C-terminal domain, as well as the uncleaved 20-kDa prohevein (53). The prohevein gene is a member of a presumed widespread gene family to which the *win* potato genes also belong (113). Prohevein and the proteins encoded by *win1* and *win2* are 65–68% homologous. The corresponding WIN1 and WIN2 proteins have not yet been isolated, so it is not known whether proteolytic processing also occurs in these proteins. The C-terminal domains of prohevein, WIN1, and WIN2 share more than 70% amino acid sequence identity with the acidic pathogenesis-related (PR) proteins, tobacco PR4 and tomato P2 (38, 57; Figure 3). However, when aligned with prohevein, WIN1, and WIN2, the tobacco PR4 and tomato P2 proteins appear to lack the hevein domain, a hinge region separating the hevein and C-terminal domain, and a 12–14-amino-acid stretch at the C-terminal end (which is also absent from WIN1). No enzymatic or biological activities have yet been ascribed to the P2 and PR4 proteins or to the C-terminal domain of hevein.

Class I Chitinases

A two-domain structure is also found in the so-called class I chitinases (110; reviewed in 72), which include basic chitinase isoforms from bean (25), tobacco (109), potato (41), poplar (85), rice (143), and a bifunctional α -amylase inhibitor/chitinase from Job's Tears (*Coix lachryma-jobi*; 4). These chitinases have an *N*-terminal 39–42-residue domain that shows approximately 50% sequence identity to the WGA domains (59). The *N*-terminal domain of these proteins is linked through a small variable glycine/proline-rich hinge region to the main chitinase domain (Figure 3). Class II chitinases show overall homology to class I chitinases, except that they lack the *N*-terminal chitin-binding domain, most of the hinge region, an internal region of the main structure, and a short 6-residue stretch at the C-terminus (58). Representatives of class II chitinases are acidic chitinases from tobacco (86), petunia (58), and barley (51). The cDNAs of class I and II chitinases encode proteins with a hydrophobic signal peptide that is not present in the mature enzymes (52, 86, 109). Class I chitinases from tobacco are thought to undergo posttranslational removal of a C-terminal heptapeptide (84). This heptapeptide has been identified as the determinant for sorting to the vacuole (84). In addition, it has been shown that tobacco class I chitinases contain a few hydroxyproline residues, which are located exclusively at the short hinge region (116). Although the significance of the prolyl hydroxylation in the class I chitinases is not known, these chitinases are the first hydroxyproline-containing proteins found in the vacuoles. The fact that both class I and II enzymes exhibit chitinase activity implies that their catalytic site is located in the main structure. The presence of the chitin-binding domain in class I chitinases confers chitin-binding properties on

them, which explains why basic (class I) chitinases but not acidic (class II) chitinases from tobacco can be purified by chitin-affinity chromatography (W. F. Broekaert, unpublished). Hence, only class I chitinases should be classified as chitin-binding proteins.

Stinging Nettle Lectin

Stinging nettle lectin (*Urtica dioica* agglutinin, UDA) is an 8.5-kDa chitin-binding protein which, like the Gramineae lectins, can agglutinate red blood cells (90). Unlike the Gramineae lectins, however, stinging nettle lectin does not effectively bind the chitin monomer, GlcNAc, which may account for its lower agglutination-specific activity (108). Amino acid sequence analysis of stinging nettle lectin has shown that this lectin has 89 residues and consists of two chitin-binding domains separated by a 4-amino-acid hinge region (8; Figures 1 and 3). The stinging nettle lectin mRNA encodes a polypeptide that extends 171 amino acids beyond the 89 amino acids of the two chitin-binding domains (56). This C-terminal domain has approximately 45% homology with class I and II chitinase catalytic domains. The identity of the C-terminal domain of prostinging nettle lectin with these other chitinase catalytic domains, however, is considerably less than the identity between the class I and class II chitinases. In spite of this, chitinase activity is observed when the 37-kDa prostinging nettle lectin or the 28-kDa C-terminal domain alone is expressed in *Escherichia coli* (56). It is not known whether prostinging nettle lectin exists as a stable protein or whether it is a transient precursor of stinging nettle lectin in the plant.

Solanaceae Lectins

A unique group of chitin-binding proteins is formed by the lectins found in some Solanaceae species, such as potato (*Solanum tuberosum*), thorn apple (*Datura stramonium*), and tomato (*Lycopersicon esculentum*). These lectins are all dimeric proteins composed of 50-kDa, 30-kDa, and 68-kDa subunits respectively (1, 17, 81). These lectins differ from all other chitin-binding proteins in that they are glycoproteins containing up to 40–50% carbohydrate (mainly arabinose). Their polypeptide chains feature at least two distinct domains. The first domain comprises a cysteine/glycine-rich sequence harboring the carbohydrate-binding site. The second domain consists of a hydroxyproline/serine-rich peptide that is extensively glycosylated (1, 37). The latter domain exists as a polyproline II helix, a similar secondary structure also found in cell wall hydroxyproline-rich glycoproteins (HRGPs; 123). In addition, the O-glycosidic arabinoside-hydroxyproline and galactoside-serine linkages found in Solanaceae lectins are identical to those in cell wall HRGPs (5). Moreover, the amino acid sequence of an internal tryptic peptide from the carbohydrate-binding domain of potato lectin matches, with six out of eight

residues, the hevein sequence at positions three to ten (16; Figure 1), and immunological cross-reactivity was observed between WGA and the Solanaceae lectins from potato and thorn apple (16). It appears, therefore, that Solanaceae lectins contain a chitin-binding domain that is linked to an HRGP-like polypeptide domain. The exact structure of these hybrid proteins, however, awaits confirmation from complete protein or cDNA sequence data.

SPATIAL EXPRESSION AND GENE REGULATION

Gramineae Lectins

The localization of WGA in wheat grain and seedlings has been studied by immunocytochemical methods (76, 97; reviewed in 96). Wheat grains contain lectin only in the embryo; no lectin has ever been detected in the endosperm, the major storage tissue of Gramineae seeds. Within the embryo, WGA is contained in the peripheral portion, i.e. in the embryonic root caps, the coleorhiza, and in outer cell layers of the radicle, coleoptile, and scutellum (76, 97). Very similar localization patterns were found for the lectins in the embryos of rye, barley, and rice although there was notable variation in lectin distribution in the coleoptiles; in wheat, lectin is located exclusively in the outer surface of the coleoptiles, barley totally lacks lectin in this organ, rye coleoptiles contain lectin in both their outer and inner surfaces, and rice coleoptiles display lectin-containing cells throughout the organ (75). The common picture arising from these studies is that only organs that come into contact with the soil during germination tend to contain lectin, at least at their periphery. In situ hybridization experiments have confirmed the particular temporal and cell-specific expression pattern of lectin genes in embryos of barley (55) and rice (132). In wheat, however, in situ hybridization performed with a probe specific for WGA isolectin B mRNA localized transcripts only in epidermal cells of the radicle, the coleorhiza, and embryonic root caps, but not in the coleoptile (95). The difference between the immunocytochemical and in situ hybridization patterns may be explained by assuming that the message for WGA lectin in the coleoptile is less stable than in the radicle and coleorhiza.

Immunocytochemistry (97) and in situ hybridization (55, 95) show that seedlings of wheat and barley express lectin only in the root caps. Root cap cells of wheat seedlings express lectin at least partially from de novo synthesized mRNA (95). At the subcellular level WGA has been shown by immunogold labeling to be located primarily in protein body vacuoles of embryonic tissues and in vacuoles of adult root cells (97). Some Gramineae, such as couch grass (*Agropyrum repens*; 30) and common reed (*Phragmites australis*; 89), also express WGA-like lectins in the leaves. The leaf-specific

lectins of couch grass, however, are similar but not identical to the lectins present in their embryos.

Several studies have shown that the synthesis of WGA is under control of the phytohormone abscisic acid (ABA). Administration of ABA accelerates the synthesis of WGA in both developing wheat embryos (99, 119) and wheat root caps (29, 98, 117). Treatment of the seedlings with fluridone, an inhibitor of carotenoid synthesis that depresses the level of endogenous ABA, causes a reduction in the amount of WGA (98). ABA-mediated induction of WGA does not involve an alteration of the spatial pattern of WGA expression; in other words, ABA enhances WGA synthesis only in those cells that already express the protein (99). Moreover, analysis of steady-state levels of WGA mRNA and nuclear run-on transcription assays has shown that ABA-mediated accumulation of WGA is attributable, at least in part, to transcriptional activation (63).

Since the key role of ABA in stress phenomena, particularly in drought stress, is well documented (10, 142), we investigated the relationship between ABA and WGA in drought-stressed and osmotically stressed wheat seedling roots. Water deficit caused a transient increase in both ABA and WGA content of the roots; WGA reached its peak level 2–3 h later than ABA. The presumed causal relationship between ABA and WGA induction was further substantiated by the fact that fluridone inhibited the stress-induced accumulation of WGA (27). The wheat seedlings were also used as a model system to study WGA induction by biological stress (28). When roots were challenged with either the pathogenic fungus *Pythium ultimum* or the saprophytic fungus *Neurospora crassa*, WGA content rose and peaked after 48 h to reach a fivefold higher level compared to uninoculated control plants. The drop in WGA content after the transient accumulation was accompanied by an increase in secretion of lectin into the imbibition medium.

Hevein and Wound-Induced Proteins

Hevein is found in rubber tree latex (3, 126), a cytoplasmic sap produced by laticifers and presumably participating in sealing off wound sites from potential invaders (36). In the latex, hevein is present in the lutoid bodies, which are vacuolar-derived organelles containing proteins (35). Analysis of hevein mRNA shows that it is present in leaves, stems, and latex. However, no hevein mRNA is detected in roots (18).

Hevein, as well as the closely related *win1* and *win2* genes from potato, belongs to a class of wound-induced genes. In young rubber tree seedlings wounded at their stems, hevein mRNA transcripts accumulated in the leaves, stems, and latex, but not in the roots (18). Analogously, *win1* transcripts increased dramatically in wounded potato leaves and stems but remained undetectable in wounded tubers and roots, while *win2* transcripts accumulated in all four tissues after wounding (113). As in the case of hevein, little or no

induction of the *win* genes was observed in wounded potato roots. The wound-induced response of hevein could be mimicked by application of the two stress-related phytohormones ethylene and ABA (18). Although the exact role of these hormones in the wound signal transduction pathway is not fully understood, it is known that endogenous levels of both ethylene (140) and ABA (88) rise upon wounding.

Stanford et al (114) studied the spatial expression of the *win2* gene in wounded tissues of transgenic potato plants that express the β -glucuronidase reporter gene under control of the *win2* promoter region. Wounding of leaves and tubers resulted initially in a strong activation of expression in cells immediately adjacent to the wound. A weaker and slower *win2* response was also observed in tissues and organs distant from the wound site, but this expression was dependent on active vascular transport, whereas the localized response itself was not.

Class I Chitinases

Cell fractionation experiments (13) and immunocytochemistry (69, 72) have shown that most class I chitinases are vacuolar proteins. One notable exception is the bean PR4 class I chitinase, which is located extracellularly (64). All class II chitinases studied so far have been characterized as extracellular enzymes (9, 54). This differential targeting between the two types of chitinases may be related to the presence or absence of a C-terminal propeptide (84; see previous section). In healthy tobacco plants, the expression of class I chitinase genes is both organ-specific and age-dependent. Highest expression levels are found in roots and developing flowers (73, 82, 109). Class I chitinase levels in the youngest top leaves are very low, whereas in the older basal leaves chitinase levels approach those in the roots (82, 109). Higher expression levels in roots vs leaves have also been noted for class I chitinases in rice (143) and *Arabidopsis thaliana* (106). In addition to being organ-specific, the expression of class I chitinases is also regulated in a cell-specific fashion. Indeed, it has been demonstrated by Keefe et al (47) that class I chitinase in healthy tobacco leaves accumulates preferentially in the epidermis.

It is well documented that class I chitinases and their corresponding transcripts are upregulated in response to various stress treatments, including wounding (15, 42, 85), ultraviolet light irradiation (15), ethylene treatment (12, 15, 25, 50), fungal (42, 66, 143) and plant cell wall elicitors (20), and fungal (42, 121, 143), bacterial (20, 33, 71) and viral infection (11, 54, 73). Unlike class II chitinases, class I chitinases in tobacco are induced only locally, not systemically, in response to tobacco mosaic virus infection (127). Moreover, tobacco class I chitinase, unlike class II chitinases, does not accumulate after treatment with salicylic acid; this is consistent with the production, upon viral infection, of salicylic acid as the systemic signal molecule

(127). In contrast, class I chitinases are turned on both locally and systemically in wounded poplar plants (85). It is possible, then, that the systemic wound response in plants is not triggered by salicylic acid but by phloem-transported peptide hormones called systemins (70, 87). Thus, tobacco class I chitinases may respond preferentially to systemin-type signal molecules rather than to salicylic acid, whereas the opposite may hold true for class II chitinases.

Several studies have addressed the spatial expression of class I chitinases in stressed plants. Ethylene treatment of tobacco leaves shifted class I chitinase expression from epidermis-specific accumulation to a more even distribution throughout the whole leaf interior (47). In ethylene-treated bean leaves, however, class I chitinase accumulation was restricted to the epidermis and to parenchyma cells adjacent to vascular strands (68). The bean chitinase promoter in transgenic tobacco also became highly activated in a zone of stem or leaf cells and around sites of fungal infection, while the activation level dropped as the distance from the infection site increased (103). This agrees with the local rather than systemic response of class I chitinase to microbial infection. The promoter sequences regulating transcriptional activation of the bean chitinase 5B gene (class I) in response to ethylene and fungal infection have been studied by deletion analysis (23, 104). At least two regions appear to play a role in the regulation of gene expression: a quantitative region that modulates the level of maximal gene expression and a region that is required for the response of gene expression to ethylene and fungal elicitors (23, 104).

Stinging Nettle Lectin

Stinging nettle lectin occurs predominantly in underground rhizomes, roots, and seeds of stinging nettle (56, 122). Immunofluorescent staining of rhizome sections has shown that the lectin is located in the outer tissues (periderm and cortex), while in roots the lectin is present exclusively in the outer periderm (D. Lambrechts and J.-P. Verbelen, unpublished). The lectin and its transcripts are not found in leaves and aerial stems of healthy plants (56, 122). Interestingly, however, aerial stems that have been buried in order to generate rhizomes accumulate the lectin to high levels (56). The developmental conversion of these stems to rhizomes must therefore induce expression of the lectin. Nothing is known so far about the factors or hormones that may govern the expression of the nettle lectin gene.

Solanaceae Lectins

Although potato lectin is normally isolated from tubers (65), it also occurs in variant forms in roots (79), fruits (48), seeds (49), and leaves (W. J. Peumans, personal communication). Casalongue & Lezica (31) presented evidence for the extracellular localization of potato lectin in tubers based on the co-sedimentation of the lectin with the cell wall pellet and on the secretion of

newly synthesized lectin from tuber slices labeled in vivo. Although they found that most of the lectin is associated with the cell walls, a fraction may also bind to the plasma membrane since tuber protoplasts can be immunostained for lectin at their surface (39). The lectin in seeds of thorn apple (*Datura* lectin) also appears to be an extracellular protein; this is evidenced by electron microscopic immunocytochemistry and by the secretion of radioactively labeled, de novo synthesized lectin polypeptides by immature seeds (21). Immunofluorescent staining of seed sections has demonstrated that the lectin is located exclusively in the outer parts, i.e. the seed coat and seed epidermis (21).

BIOLOGICAL ACTIVITIES

Antifungal Activity

Class I chitinases are the chitin-binding proteins whose antifungal properties have been best characterized. Schlumbaum et al (107) first reported on growth inhibition of a fungus, *Trichoderma viride*, caused by a class I chitinase from bean leaves. Since then, others have demonstrated antifungal effects of chitinases from other plants, such as wheat (102), barley (52, 102), maize (46, 102), tobacco and thorn apple (17), and pea (67). Many plant pathogenic fungi are inhibited by chitinases only when applied in combination with β -1,3-glucanases (60, 67). This is not surprising since the cell walls of most fungi are mainly composed of chitin and β -glucans (130). Synergistic antifungal effects have also been reported for combinations between chitinase, β -1,3-glucanase and ribosome-inactivating protein (52), between chitinase and a thaumatin-like protein (43), and between chitinase and stinging nettle lectin (22). It is not known at present whether the antifungal effect of chitinases results from inhibition of chitin synthesis or from hydrolysis of preformed chitin chains in the cell wall. Both inhibition of chitin synthesis due to breakdown of nascent chitin chains (77) and release of chitin fragments from isolated fungal cell wall (12, 67, 141) have been demonstrated in vitro using purified enzymes.

There has been an ongoing controversy over whether chitin-binding lectins can act as antifungal proteins, as do chitinases. As early as 1975, Mirelman et al (74) found that WGA inhibited spore germination and hyphal growth of *T. viride*. In addition, other chitin-binding lectins were demonstrated to possess antifungal activity. Callow (26) reported that potato lectin inhibited hyphal extension and spore germination of *Botrytis cinerea*. This lectin also reportedly lyses zoospores of *Phytophthora infestans* (2, 40). Bramble & Gade (14) found that the chitin-binding lectin from pokeweed retards germ tube elongation of *Neurospora crassa* and enhances the susceptibility to hypotonic lysis of *Botryodiplodia theobromae* germ tubes. However, the current view is that

at least some of these results are attributable to chitinases present as contaminants in the lectin preparations. Schlumberg et al (107) completely abolished the antifungal activity of commercial preparations of potato lectins and tomato lectin by addition of antiserum against bean chitinase. Moreover, chitinase-free preparations of WGA, pokeweed lectin, and potato lectin failed to inhibit growth of *T. viride* (107), as well as seven other tested fungi (22; W. F. Broekaert, unpublished).

Three newly characterized nonenzymatic chitin-binding proteins—stinging nettle lectin, hevein, and the Ac-AMPs—have recently been shown to exhibit antifungal properties different from those of the chitinases (22, 124, 19, respectively). Chitinase-free preparations of nettle lectin caused 50% inhibition of germ tube growth at concentrations varying from 20–150 µg/ml. A chitin-negative fungus, *Phytophthora erythroseptica*, was not affected by the nettle lectin. Hevein has antifungal properties similar to nettle lectin but is, on average, three to five times less active (124). The Ac-AMPs are the most potent of the chitin-binding proteins analyzed to date. Concentrations required for 50% inhibition of fungal growth vary between 1–10 µg/ml. Moreover, their activity spectrum is much broader than that of stinging nettle lectin or chitinases (19). The Ac-AMPs also exert inhibitory activity toward Gram-positive but not Gram-negative bacteria (19).

The mechanism of growth inhibition by the small-sized chitin-binding proteins is not fully understood. Stinging nettle lectin, the largest of these proteins, is only 8.5-kDa, while hevein is 5-kDa and the Ac-AMPs are approximately 6-kDa. The Gramineae lectins, on the other hand, form dimers of 36-kDa and do not show antifungal activity (22, 107). It is tempting to speculate that the small size of the chitin-binding proteins exhibiting antifungal activity allows them to penetrate into and interact with the fungal wall, whereas the Gramineae lectins cannot. An estimate of fungal wall pore size supports this hypothesis (78). This study predicts that proteins larger than 15–20-kDa will not be able to pass through the fungal cell wall. Both nettle lectin and hevein are below the size exclusion limit of the fungal cell wall and therefore can penetrate the wall pores to reach their target site. A preliminary study on the mode of action of stinging nettle lectin on the fungus *Phycomyces blakesleeana* has indicated that the cell wall composition of lectin-treated hyphae is altered: The chitin content is increased relative to untreated controls (125). This alteration may result in enhanced cell wall plasticity and thus interfere with the apical growth process, which is dependent on the mechanical strength of the hyphal cell wall (129).

The potential for enhancing plant resistance against fungal pathogens by constitutive expression of class I chitinases has been addressed by two groups. Broglie et al (24) found that tobacco (*Nicotiana tabacum* cv. Xanthi) and canola (*Brassica napus* cv. Westar) expressing a bean chitinase gene under

control of the cauliflower mosaic virus 35S promoter had an increased ability to survive in soils infected with *Rhizoctonia solani* and showed delay in disease symptom development. However, Neuhaus et al (83) found that high levels of a tobacco (*N. tabacum*) class I chitinase gene expressed in *Nicotiana sylvestris* did not enhance resistance to *Cercospora nicotiana*. Thus, these studies are inconclusive as to the general effectiveness of engineering fungal resistance in plants by overexpressing chitinase alone. Since stinging nettle lectin acts synergistically with chitinase to inhibit fungal growth in vitro, the possibility exists that overexpression of prostinging nettle lectin, with its lectin and chitinase domains, will enhance resistance to fungal pathogens not seen with the chitinase alone. Another possibility is that stinging nettle lectin will work cooperatively with endogenous chitinases in the transgenic plant.

Insect Antinutrient Activity

Recently, the effect of several chitin-binding lectins on insect larvae has been examined using artificial seed diet systems. WGA appears to be among the most potent of the lectins studied so far (34, 80). When fed at 10 mg per g diet to cowpea weevil larvae (*Calosobruchus maculatus*), WGA increased the time of larva development from 32 to 55 days (80). Moreover, WGA produced 50% mortality of two major corn pests, European corn borer (*Ostirinia nubilalis*; 34) and Southern corn rootworm (*Diabrotica undecimpunctata*; T.H. Czapla, personal communication) when incorporated in their diet at 0.59 mg/g and 3 mg/g respectively. All three WGA isolectins have similar deleterious effects on cowpea weevil larvae (44). Similar results have now been obtained with rice lectin and stinging nettle lectin (45). However, purified Solanaceae lectins

Table 1 Summary of in vitro biocidal activities of chitin-binding proteins

	Antifungal activity	Reference	Insecticidal activity	Reference
WGA	— ^a	22, 107	+ ^b	34, 44, 80
Rice Lectin	ND ^c		+	45
<i>Datura</i> Lectin	—	16	+	d
Tomato Lectin	—	107	+	d
UDA	+	22	+	45
Hevein	+	124	ND	
Ac-AMP	+	19	ND	
Class I Chitinase	+	17, 46, 52, 67, 102, 107	ND	

^ano activity

^bactivity

^cnot determined

^dL. L. Murdock and J. E. Huesing, personal communication

(thorn apple and tomato lectins) have significantly less effect on developing cowpea weevil larvae (L. L. Murdock and J. E. Huesing, personal communication). The effect on insects of other chitin-binding proteins, such as chitinases, hevein, and WIN proteins, remains to be determined.

Recently, a cDNA clone for WGA was introduced into embryogenic maize suspension cells, and plants were regenerated (61). Preliminary studies with transformed callus and transformed plants indicated that expression of this gene in maize tissue may be a potential means of improved tolerance to European corn borer (61).

Although the mode of action of the chitin-binding lectins is not known, it is tempting to speculate that their deleterious effect on insect development is mediated by the binding of these lectins to chitin in the peritrophic membrane of the insect midgut. This membrane, which is composed primarily of chitin and proteins (101), protects the midgut epithelium from abrasive food particles. By binding to components on this membrane, the lectins may alter its physical properties or prevent the formation of the membrane itself. The plant defense properties of the proteins in the chitin-binding family are summarized in Table 1.

EVOLUTIONARY CONSIDERATIONS

The presence of highly conserved chitin-binding domains in different classes of proteins suggests that they arose as a result of gene duplications and/or gene fusions from a common ancestral gene encoding a single chitin-binding domain. This ancestral gene has been thought to resemble hevein (110, 138). However, it is now clear that the hevein gene does not encode a one-domain protein (18). Shinshi et al (110) proposed an attractive model for the evolution of class I chitinases whereby a chitin-binding domain sequence was introduced into the coding region of an ancestral chitinase gene by a transposition event. The mechanism of insertion may, as in the case of transposon insertion (105), involve an asymmetric cut of the target DNA strands, which produces staggered ends; fill-in reactions, insertion of the sequence, and ligation followed, thereby creating a direct repeat at both ends of the introduced sequence. Imperfect direct 9–10 bp repeats flanking the chitin-binding domain sequence are present in different class I chitinase genes, as well as in the potato *win* genes (110) and in the hevein cDNA (18; see nucleotide positions 51–60 and 210–219). The reason for the apparent mobility of the chitin-binding domain sequence in the plant genome is not understood, but it clearly differs significantly from true transposons. The chitin-binding domain sequence does not possess indirect repeats at its termini (110), which are typical of transposons (105). However, the sequences bordering the chitin-binding domain may be hot spots for nicking events, which lead either to excision and reinsertion

elsewhere in the genome or to self-duplication by fill-in of single-stranded, staggered ends. The latter mechanism may explain how an ancestral sequence has given rise to genes with multidomain structures such as those of Gramineae lectins and nettle lectin. Wright et al (139) derived an evolutionary tree based on a comparison of the sequences of Gramineae lectins, stinging nettle lectin, class I chitinases, and hevein and WIN proteins. The tree contains two major branches: One branch includes the four-domain Gramineae lectins and the two-domain stinging nettle lectin; the other branch clusters all sequences of proteins with a single chitin-binding domain. The tree topology is consistent with a proposed history of two subsequent duplications from a single domain ancestor to a two-domain intermediate and from the two-domain intermediate to a four-domain protein.

CONCLUSIONS

The biological function of chitin-binding proteins in plants has yet to be conclusively demonstrated. There is, however, a wealth of circumstantial evidence for functions in plant defense, without excluding additional functions in plant development. First, there is the lack of chitin in plants and its presence in many plant pathogens and pests. Second, the conservation of amino acid sequence and chitin-binding activity within this family of proteins indicates an essential role in plant interactions with organisms containing chitin. Especially noteworthy is the distinctive pattern of cysteine residues and the conservation of residues directly involved in chitin binding (see Figure 1). Fusion of the chitin-binding domain to a number of structurally unrelated domains suggests that it may be genetically recombined with other polypeptides by the plant to create fusion proteins with novel properties. The evolutionary advantage of such a hybrid protein may be that it can be targeted more specifically to chitin-containing surfaces, such as the insect midgut or the fungal cell wall. It is interesting that class I chitinases from tobacco have higher specific chitinolytic activities than their class II counterparts (54). We have also shown that an artificial fusion protein consisting of the 43-residue hevein domain and a bacterial maltose-binding protein exhibits affinity toward chitin, whereas the maltose-binding protein itself does not (53). In some cases (e.g. hevein and most likely stinging nettle lectin), the fusion proteins are proteolytically cleaved into two proteins, each presumably with its own biological activities (53, 56).

The localization pattern of chitin-binding proteins in plants also suggests that they interact with plant pathogens. The Gramineae lectins are expressed in peripheral tissues that first expand into new areas of soil as the embryo germinates and the root system grows. Cell-specific localization patterns in peripheral tissues of plant organs have also been reported for class I chitinases

in tobacco leaves (47, 68), thorn apple lectin in seeds (21), and stinging nettle lectin in roots and rhizomes (22). The preferential accumulation of these proteins in tissues that are in direct contact with the stress source is consistent with their postulated defense function. The vacuolar localization of most chitin-binding lectins (except Solanaceae lectins) suggests that these proteins act after the invasion of the plant tissue and release of the cellular content.

Induction of chitin-binding protein expression by wounding, microbial infection, and the plant stress hormones also indicates that these proteins act as part of the plant defense mechanism. Chitinases, Gramineae lectins, hevein, and WIN proteins are all induced under these conditions. Finally, and perhaps most importantly, are the *in vitro* antifungal and insecticidal activities demonstrated for chitin-binding proteins. Indeed, every chitin-binding protein tested exhibits either insecticidal or antifungal activities or both. Moreover, the first results obtained with transgenic plants overexpressing class I chitinases or Gramineae lectins indicate that these proteins can contribute to resistance against fungal diseases (24) or insect attack (61).

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