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Gastrodianin-like mannose-binding proteins: a novel class of plant proteins with antifungal properties

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Summary

The orchid *Gastrodia elata* depends on the fungus *Armillaria mellea* to complete its life cycle. In the interaction, fungal hyphae penetrate older, nutritive corms but not newly formed corms. From these corms, a protein fraction with *in vitro* activity against plant-pathogenic fungi has previously been purified. Here, the sequence of gastrodianin, the main constituent of the antifungal fraction, is reported. Four isoforms that encoded two different mature proteins were identified at the cDNA level. Another isoform was detected in sequenced peptides. Because the antifungal activity of gastrodianins produced in and purified from *Escherichia coli* and *Nicotiana tabacum* was comparable to that of gastrodianin purified from the orchid, gastrodianins are the active component of the antifungal fractions. Gastrodianin accumulation is probably an important part of the mechanism by which the orchid controls *Armillaria* penetration. Gastrodianin was found to be homologous to monomeric mannose-binding proteins of other orchids, of which at least one (*Epipactis helleborine* mannose-binding protein) also displayed *in vitro* antifungal activity. This establishes the gastrodianin-like proteins (GLIPs) as a novel class of antifungal proteins.

Keywords: antifungal protein, *Epipactis helleborine*, *Gastrodia elata*, lectin, mannose-binding protein, Orchidaceae.

Introduction

A possible approach to engineer resistance towards fungal pathogens in crops is the transgenic production of proteins with antifungal properties. Pyramiding genes for such proteins is expected to result in broad-range, durable resistance. In the last decade, several classes of proteins that retard the growth of fungi in *in vitro* assays have been identified. Examples include pathogenesis-related proteins, such as PR-1-type proteins (Niderman *et al.*, 1995), PR-2-type proteins (for instance, β-1,3-glucanases; Mauch *et al.*, 1988), chitinases of the PR-3-type (Schlumbaum *et al.*, 1986) and PR–11-type (Melchers *et al.*, 1994), PR-4-type proteins (Hejgaard *et al.*, 1992), PR-5-type proteins (Roberts and Selitrennikoff, 1990), hevein-type proteins (for example, *Urtica dioica* agglutinin; Broekaert *et al.*, 1989), non-specific lipid transfer proteins (Terras *et al.*, 1992a), thionins (Stuart and Harris, 1942), plant defensins (Terras *et al.*, 1992b) and type-I ribosome-inactivating proteins (Leah *et al.*, 1991; Roberts and Selitrennikoff, 1990). Proteins with *in vitro* antifungal activity that are not structurally related to one of the above classes are a plant cystatin (Pernas *et al.*, 1999) and several cysteine-rich plant proteins including Mj-AMP from

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                                CAAAGTAGCACAGGTATTCCACCTAGCCATCAAGCAGCC

    ATG GCA GCA TCC GCA AGC ACT GCG GTA ATC CTG TTC TTT GCC GTG
1   M   A   A   S   A   S   T   A   V   I   L   F   F   A   V
    ACA ACA ATG ATG AGT TTG TCA GCC ATC CCG GCC TTC GCT TCA GAC
16  T   T   M   M   S   L   S   A   I   P   A   F   A   S   D
    CGT TTG AAT TCC GGC CAC CAA CTT GAT ACC GGG GGC TCA CTA GCA
31  R   L   N   S   G   H   Q   L   D   T   G   G   S   L   A
    CAA GGC GGC TAC CTA TTC ATA ATA CAA AAC GAT TGT AAT CTT GTC
46  Q   G   G   Y   L   F   I   I   Q   N   D   C   N   L   V
    TTA TAT GAT AAC AAC AGA GCG GTC TGG GCA TCA GGA ACC AAC GGA
61  L   Y   D   N   N   R   A   V   W   A   S   G   T   N   G
    AAG GCC TCC AAC TGC TTC CTT AAG ATG CAG AAT GAT GGC AAC CTC
76  K   A   S   N   C   F   L   K   M   Q   N   D   G   N   L
    GTT ATT TAT AGC GGT AGC AGG GCA ATA TGG GCA AGC AAC ACC AAT
91  V   I   Y   S   G   S   R   A   I   W   A   S   N   T   N
    CGC CAA AAC GGT AAC TAC TAT CTG ATC CTT CAG AGA GAT CGT AAC
106 R   Q   N   G   N   Y   Y   L   I   L   Q   R   D   R   N
    GTC GTC ATA TAC GAT AAT TCT AAT AAT GCG ATT TGG GCA ACC CAC
121 V   V   I   Y   D   N   S   N   N   A   I   W   A   T   H
    ACC AAC GTT GGA AAT GCT GAA ATC ACT GTC ATC CCA CAC AGC AAC
136 T   N   V   G   N   A   E   I   T   V   I   P   H   S   N
    GGC ACA GCG GCG GCG TCT GGC GCA GCA CAG AAC AAG GTC AAT GAA
151 G   T   A   A   A   S   G   A   A   Q   N   K   V   N   E
    TTA TAC ATA TCC ATG TAC TAG CGGTCTAAGAGAATAGCTAGCTAGCTAGCTA
166 L   Y   I   S   M   Y   *
    TGCGCGCATGCGTCTGTTCTCTGTGCGTGCACAAGAATAAAATCTGTTGAGGTGTTGGG

    ATGTTCATATTAAATAAATTAAGCTGCTTTATAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. cDNA sequence and deduced amino acid sequence of gastrodianin-MNF. The amino acid sequence of the mature protein is in bold with positions at which the isoforms differ underlined. Polyadenylation sites are indicated in italics.

Mirabilis (Cammue *et al.*, 1992), MiAMP1 from *Macadamia* (Marcus *et al.*, 1997), MBP-1 from maize (Duvick *et al.*, 1992), Ib-AMP from *Impatiens* (Tailor *et al.*, 1997) and snakin-1 from potato (Segura *et al.*, 1999). For a number of these proteins, expression of the corresponding genes in transgenic plants has resulted in enhanced tolerance towards one or more fungi. Examples are PR-1 (Alexander *et al.*, 1993), β-1,3-glucanases (Yoshikawa *et al.*, 1993), chitinases (Broglie *et al.*, 1991), PR-4 (Lee and Raikhel, 1995), PR-5 (Liu *et al.*, 1994), thionins (Epple *et al.*, 1997), defensins (Terras *et al.*, 1995), type-I ribosome-inactivating proteins (Logemann *et al.*, 1992) and Mj-AMP (De Bolle *et al.*, 1996).

The orchid *Gastrodia elata* Bl. F. *flavida* S. Chow is a traditional Chinese medicinal herb. *G. elata* is devoid of chlorophyll and leads a parasitic life on the fungus *Armillaria mellea*. During its development, *Armillaria* invades the so-called nutritive or primary corm of the orchid. The fungal hyphae are arrested and digested in the cortical layer. Infection is a condition for the formation of a secondary, terminal corm that will produce flowers. It is assumed that nutrients released upon digestion are transported from the nutritive corm into the terminal corm to sustain the development of the latter (Yang and Hu, 1990). The terminal corm is completely resistant to fungal infection. Hu *et al.* (1988) were the first to purify, from the cortex of the terminal corm, a protein (*Gastrodia* antifungal protein or GAFP, also known as gastrodianin) that retarded the progression of the hyphal growth of a fungus (*Trichoderma*) *in vitro*. Fungal infection induces gastrodianin production and the protein accumulates to high levels in the cortex of the terminal corm (Hu and Huang, 1994). Although chitinases and β-1,3-glucanases are present in *Gastrodia* corms, they are less active than gastrodianin in *in vitro* assays (Yang and Hu, 1990), and active gastrodianin preparations are free of chitinase and β-1,3-glucanase activity (Hu *et al.*, 1988). Gastrodianin inhibits spore germination and retards hyphal elongation (Xu *et al.*, 1998). Besides *Trichoderma*, gastrodianin inhibits the phytopathogens *Armillaria mellea* (Hu and Huang, 1994), *Rhizoctonia solani*, *Valsa ambiens*, *Gibberella zeae*, *Ganoderma lucidum* and *Botrytis cinerea* (Xu *et al.*, 1998) *in vitro*. Molecular mass estimates for gastrodianin range between 10 kDa (Xu *et al.*, 1998) and 14 kDa (Hu *et al.*, 1988). Gastrodianin is a non-agglutinating, monomeric, mannose- and chitin-binding protein with an N-terminal sequence that is homologous to known lectins of the Orchidaceae (Xu *et al.*, 1998).

Here, we have (i) elucidated the sequence of gastrodianin, (ii) isolated and expressed the corresponding cDNAs in *E. coli* and *Nicotiana tabacum*, (iii) purified the recombinant protein from these expression systems and (iv) assessed the antifungal activity of the recombinant protein as such and in comparison to other proteins in *in vitro* assays.

Results

N-terminal peptide sequencing

Gastrodianin was purified from *Gastrodia elata* corms as described previously (Xu *et al.*, 1998). The purified protein fraction resulted in a single band with an estimated molecular mass of 12 kDa when analyzed on SDS-PAGE. Amino acid sequencing confirmed the previously determined (Xu *et al.*, 1998) N-terminal sequence of the protein, being SDRLNSGHQLDTGGSLAQGGYLFI.

cDNA cloning

Based on the N-terminal sequence, two degenerate primers were designed for (nested) rapid amplification of cDNA ends (RACE). The outer primer (primer 1) corresponded to the first nine N-terminal amino acids of the sequenced N-terminal peptide. The inner primer (primer 2) covered amino acids 16–24 of the same peptide. Marathon adaptor-primers 1 and 2 were used in combination with the inner and outer primers to amplify cDNA fragments from adaptor-ligated cDNA. Fragments could be amplified that matched previously sequenced internal peptides. A 3′ untranslated region of 121 bp with two polyadenylation sites was found downstream from the stop codon.

The use of degenerate primers for the amplification of 5′ cDNA fragments was not successful. Such primers consistently yielded a sequence (EMBL accession number AJ278421) that was significantly homologous to gastrodianin (as deduced from 3′ RACE) but that clearly differed at the N-terminus. Therefore, specific primers that could be deduced from the obtained 3′ RACE fragments (primers 3 and 4) were used. Fragments matching the known gastrodianin sequences were obtained. Assuming that translation is initiated at the first ATG codon, comparison of the 5′ RACE sequences with the N-terminal peptide revealed a 28-amino-acid signal peptide, which is in accordance with a predicted signal peptide-processing site between A_{28} and S_{29}, based on the rules of von Heijne (1986). Amplification with different gene-specific primers gave fragments that were identical, except at position 18 (Figure 1), where either an M or a V was found, and at positions 79 and 81, where either $G_{79}M_{81}$ or $N_{79}F_{81}$ was encountered. The longest 5′ cDNA obtained contained a 41-bp 5′ untranslated region.

Based on the 3′ and 5′ RACE fragments, 3′ and 5′ gene-specific primers for gastrodianin were designed. Four types of full-length cDNA clones were amplified that only differed at the amino acid positions 18, 79 and 81 (Figure 1). Depending on the occurrence of different amino acids at positions 18, 79 and 81, the gastrodianin isoforms will further be referred to as gastrodianin-MNF, gastrodianin-MGM, gastrodianin-VGM and gastrodianin-VNF (EMBL accession numbers AJ277783, AJ277784, AJ277785 and AJ277786).

In 13 independently amplified cDNAs, gastrodianin-MGM, gastrodianin-MNF, gastrodianin-VGM and gastrodianin-VNF were present at a ratio of 3/13, 3/13, 2/13 and 5/13, respectively. At the processed protein level, only two isoforms could be distinguished (position 18 being located in the signal peptide), further called gastrodianin-NF and gastrodianin-GM.

A database search with BlastP 2.0.6 (Altschul *et al.*, 1997) showed that gastrodianins are members of the superfamily of the monocot mannose-binding lectins that have been isolated from species of the Orchidaceae, Amaryllidaceae, Alliaceae, Liliaceae and Araceae (Barre *et al.*, 1996). The amino acid sequence of mature gastrodianin-NF was 85% and 83% identical to that of the monomeric mannose-binding lectins from the orchids *Listera ovata* (twayblade; Van Damme *et al.*, 1994a) and *Epipactis helleborine* (broad-leaved helleborine; Van Damme *et al.*, 1994a), respectively. Identities between 45% and 60% exist between gastrodianins and the multimeric lectins of for example *L. ovata* (Van Damme *et al.*, 1994b), *E. helleborine* (Van Damme *et al.*, 1994b), *Cymbidium* (Van Damme *et al.*, 1994b), *Galanthus nivalis* (snowdrop; Van Damme *et al.*, 1991), *Allium* (Van Damme *et al.*, 1993), *Polygonatum* (Van Damme *et al.*, 1996a), *Tulipa* (Van Damme *et al.*, 1996b) and *Clivia* (Van Damme *et al.*, 1994c).

Mass spectrometry (MS) analysis

The mass of the purified mature protein, as determined by electrospray MS, was 12 399 Da. Considering that the N-terminal residue is S_{29}, N_{140} can be predicted to be the C-terminal residue. This assumption was confirmed by MS analysis. The protein was digested with trypsin and the isolated peptides were analyzed by nanospray MS (Wilm and Mann, 1996). Nearly the entire sequence of gastrodianin was recovered in a single analysis (except for the longest, 30-residue peptide). Among the tryptic peptides, a peptide with a mass of 2317 Da and a MS/MS spectrum corresponding with the sequence NVVIYDNSNNAIWAT-HTNVGN was identified. Because this tryptic peptide did not end on K or R, it was probably the C-terminal tryptic peptide and, thus, N_{140} is most probably the C-terminal end residue of the mature protein. In the mixture of tryptic peptides, only the ion with mass 782.3 Da and with MS/MS spectrum of the sequence ASNCFLK was present and not the ion with mass 709.3 Da (sequence ASGCMLK), indicating that the purified protein predominantly contained gastrodianin-NF. The theoretical molecular mass of gastrodianin-NF with N_{140} as the C-terminal residue is 12 401.5 Da, which is in good agreement with a mass of 12 399 Da as determined by electrospray MS. On the other hand, a peptide containing K rather than N at position 108 (Figure 1) was detected, indicating that *G. elata* produced at least one isoform of gastrodianin of which the cDNA was not identified.

Activity of gastrodianin produced in E. coli

The part of the cDNAs that encodes the mature isoforms gastrodianin-NF and gastrodianin-GM was translationally fused to the maltose-binding protein (MBP) in the vector pMal-p2 to produce fusion proteins in *E. coli*. Cleavage of these fusion proteins with factor Xa was expected to release proteins that exactly matched the predicted amino

acid sequence of mature gastrodianin. In addition, the fusion protein was targeted to the periplasmic compartment, allowing disulfide bonds to be formed. MBP-gastrodianin-NF and MBP-gastrodianin-GM fusion proteins were prepared, digested with factor Xa and concentrated in 50 mM NaAc (pH 5) buffer. It was first established that incubation of native gastrodianin (purified from *G. elata*) with factor Xa did not affect the antifungal activity (data not shown). Digested fusion proteins containing 0.8 μg gastrodianin-NF (Figure 2, number 1) or gastrodianin-GM (data not shown) inhibited mycelial growth of *V. ambiens*. Gastrodianin-NF and gastrodianin-GM had a comparable level of activity (data not shown). As a negative control, MBP was treated in exactly the same way (incubation with factor Xa and concentration) as the fusion proteins and had no effect on fungal growth (Figure 2, number 2), excluding that MBP in itself, factor Xa, or contaminants copurified from *E. coli*, caused fungal growth inhibition. The activity of the recombinant proteins was similar to that of native gastrodianin isolated from *G. elata* (Figure 2, numbers 3–6). As opposed to the cleaved fusion proteins, intact fusion proteins had no inhibitory activity when tested at the same molar concentration (data not shown). It should be noted that the activity varied between independently prepared *G. elata* samples (Figure 2, numbers 3/4 versus numbers 5/6). Clearly, results obtained with the type of assays utilized here should be interpreted in a qualitative rather than a quantitative fashion.

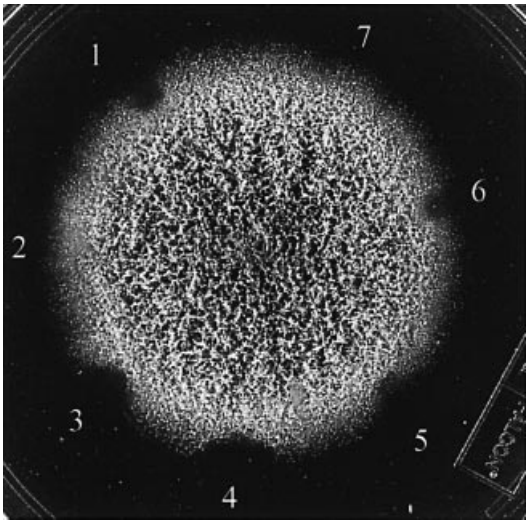

Figure 2. *Valsa ambiens* growth inhibition assay with recombinant gastrodianin produced in *E. coli*.
(1) MBP-gastrodianin-NF digested with factor Xa. The sample contains 0.8 μg gastrodianin. (2) MBP in equivalent amount and treated in the same way as the MBP-gastrodianin-NF sample. (3,4) 0.8 μg and 2.5 μg gastrodianin isolated from *G. elata* corms, respectively. (5,6) 0.2 μg and 0.5 μg from an independent preparation of gastrodianin from *G. elata* corms, respectively. (7) 50 mM NaAc (pH 5).

Activity of gastrodianin produced in N. tabacum

Tobacco was transformed with *Agrobacterium tumefaciens* carrying the binary plasmid pVNFbin. This plasmid contained the gastrodianin-VNF-encoding sequence under the control of the 35S promoter and an Ω leader sequence. Gastrodianin as detected by protein gel blot analysis in extracts of transgenic plants had a mobility corresponding to 12 kDa, indicating that processing in tobacco occurred essentially as in *G. elata*. Production levels were assessed by protein gel blot analysis. From Figure 3, a rough estimate of the level of gastrodianin can be deduced. By visual comparison with the band produced from 0.1 μg purified gastrodianin (lane 1), several plants represented in Figure 3 produced at least 0.1 μg gastrodianin per 30 μg total protein (the amount of protein loaded in each lane), corresponding to an accumulation level of 0.3% or more.

Gastrodianin was purified from leaves of highly expressing primary transformants by ion exchange and affinity chromatography and was pure, as judged from a Coomassie blue-stained polyacrylamide gel. No protein was recovered when the same procedure was applied to tobacco plants transformed with the control vector pAVAT. Recombinant protein from tobacco inhibited growth of *V. ambiens* (Figure 4, numbers 1/2). The activity level was in the same range as that obtained with gastrodianin purified from *G. elata* corms (Figure 4, numbers 3–7).

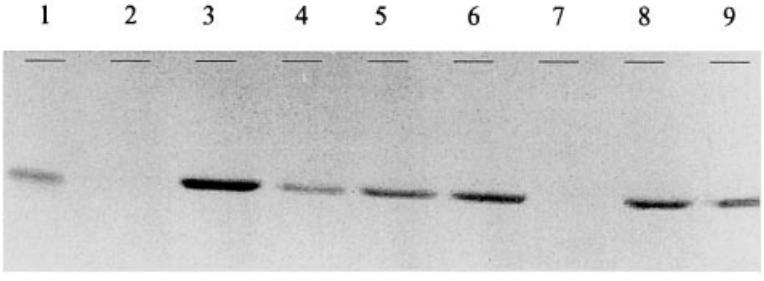

Figure 3. Protein gel blot analysis of transgenic tobacco plants.
Lane 1, 0.1 μg gastrodianin purified from the orchid; lane 2, non-transgenic plant; lanes 3–9, transgenic plants.

Activity of gastrodianin relative to other proteins

The antifungal activity of gastrodianin was compared with that of *Urtica dioica* agglutinin (UDA) (Peumans *et al.*, 1984), a protein with well characterized antifungal activity (Broekaert *et al.*, 1989; Does *et al.*, 1999). UDA (Figure 5, numbers 1–4) had a growth inhibitory activity towards *V. ambiens* that was comparable to that of gastrodianin purified from either *G. elata* (Figure 5, numbers 6/7) or from transgenic tobacco (Figure 5, number 5). Because gastrodianins showed considerable homology to several monocot mannose-binding lectins, 5 μg of the (monomeric) merolectin of *E. helleborine* (Van Damme *et al.* 1994a), the dimeric hololectins of *E. helleborine* and *Cymbidium* (Van Damme *et al.*, 1994b) and the tetrameric hololectins of *P. multiflorum* (Van Damme *et al.*, 1996a) and of *G. nivalis* (Van Damme *et al.*, 1987) were applied to *V. ambiens*-inoculated plates. Only the merolectin of *E. helleborine* had antifungal activity (Figure 6). This protein had, among the proteins tested, the highest degree (83% at the amino acid level) of identity with gastrodianin-NF.

Discussion

Four different full-length cDNA sequences that matched peptide sequences from an antifungal fraction of *Gastrodia elata* corms were obtained. The gastrodianin cDNAs differed at three amino acid positions. Comparison with the N-terminal peptide sequence and the C-terminal residue of protein fractions purified from the orchid revealed that gastrodianins are formed as preproproteins from which, upon maturation, a 31-amino acid C-terminal peptide and a 28-amino acid N-terminal signal peptide are removed. The resulting mature proteins are 112 amino acids long and have, depending on the isoform, a theoretical molecular mass of between 12 328.5 Da (gastrodianin-GM) and 12 401.5 Da (gastrodianin-NF). The four cDNAs encode, at the mature protein level, two isoforms that differ in two residues. Only one isoform (gastrodianin-NF) was actually found in sequenced peptides from the active fraction. On the other hand, peptide sequencing of that fraction yielded yet another isoform for which no corresponding cDNA was detected. It is not clear whether the isoforms reflect allelic or nonallelic variation because the mRNA and protein samples used were derived from tissues of pooled plants, potentially of different genotypes.

Gastrodianins share the highest degree of homology with the monomeric lectins of the orchids *Epipactis helleborine* and *Listera ovata* (Van Damme *et al.*, 1994a). They are also homologous to many lectins of the Orchidaceae, Amaryllidaceae, Alliaceae, Liliaceae and Araceae (Barre *et al.*, 1996; Van Damme *et al.*, 1998), including the insecticidal *Galanthus nivalis* agglutinin (GNA) (Hilder *et al.*, 1995; Van Damme *et al.*, 1987), to the sweet-tasting and taste-modifying curculin from *Curculigo latifolia* (Abe *et al.*, 1992) and to the vesicle-binding comitin from *Dictyostelium discoideum* (Stratford and Brown, 1985).

The crystal structure of GNA has been elucidated (Hester *et al.*, 1995). Like GNA, gastrodianins display a triple

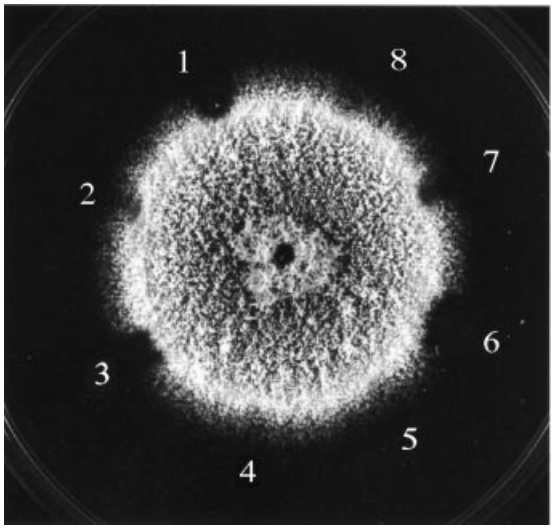

Figure 4. *V. ambiens* growth inhibition assay with recombinant gastrodianin isolated from transgenic tobacco.
(1,2) 5 μg and 1.25 μg gastrodianin purified from transgenic tobacco, respectively. (3–5) 5 μg, 2 μg and 0.5 μg gastrodianin purified from *G. elata* corms, respectively. (6,7) 2 μg and 0.5 μg from an independent preparation of gastrodianin from *G. elata*, respectively. (8) 50 mM NaAc (pH 5).

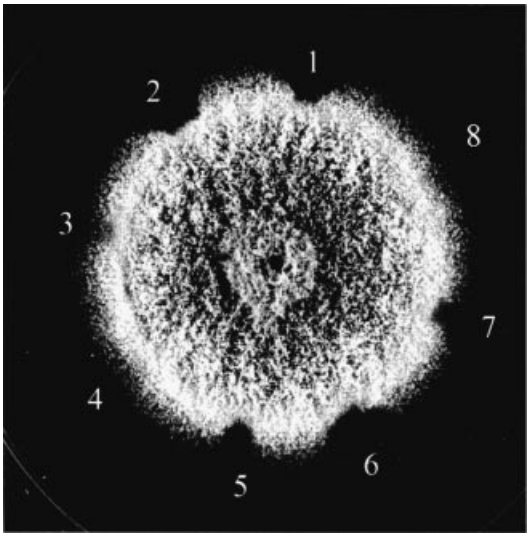

Figure 5. *V. ambiens* growth inhibition by gastrodianin and UDA.
(1–4) 5 μg, 2 μg, 0.5 μg and 0.1 μg UDA, respectively. (5) 5 μg gastrodianin isolated from transgenic tobacco. (6,7) 2 μg and 0.5 μg gastrodianin isolated from *G. elata*, respectively. (8) 50 mM NaAc (pH 5).

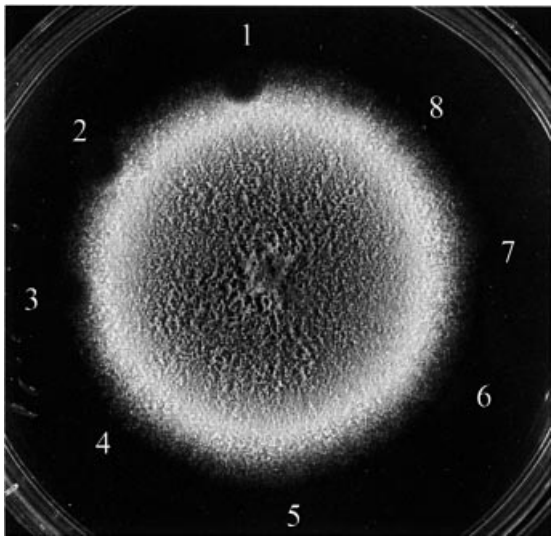

Figure 6. *V. ambiens* growth inhibition by gastrodianin and related proteins.
(1,2) 5 μg and 1.5 μg gastrodianin isolated from *G. elata,* respectively. (3,4) 5 μg *Epipactis helleborine* merolectin and dimeric hololectin, respectively. (5) 5 μg GNA. (6) 5 μg *Polygonatum* tetrameric hololectin. (7) 5 μg *Cymbidium* dimeric hololectin. (8) 50 mM NaAc (pH 5).

pseudo-repeat (residues G_{47} to N_{74}, S_{78} to N_{105} and G_{109} to N_{137}; Figure 1) that in GNA gives rise to three sequential, four-stranded, β-sheet subdomains arranged as a barrel. The conservation between gastrodianins and GNA of residues that contribute to the hydrophobic barrel core and of two cysteine residues that potentially form an intramolecular disulfide bridge, suggests that gastrodianins and GNA share the same fold. In agreement with the observation that gastrodianins strongly bind to mannose and chitin (Xu *et al.*, 1998), all residues that were shown to be involved in mannose binding in GNA (Hester *et al.*, 1995) are also present in gastrodianin.

Expression of gastrodianin cDNAs in *E. coli* resulted in the production of proteins with an antifungal activity that is comparable to that of native protein samples purified from orchid corms. Thus, gastrodianins account for most, if not all, of the antifungal principle of the purified samples. Conceivably, gastrodianin accumulation plays an important role in the mechanism by which *G. elata* controls *A. mellea* penetration.

When tested against the fungus *V. ambiens*, the activity of gastrodianin was on a par with that of an established antifungal protein, UDA. Interestingly, the mannose-binding monomeric lectin of *E. helleborine*, which is significantly homologous to gastrodianins, also retarded hyphal progression. This observation establishes the gastrodianin-related monomeric lectins of orchids as a novel class of antifungal proteins, further called gastrodianin-like proteins (GLIPs). Lectins of different types provide plants with a passive defense system towards pathogens as diverse as bacteria, fungi, insects and higher animals (Peumans and Van Damme, 1995). From the ancestors of the superfamily of monocotyledonous mannose-binding lectins, defense proteins with different specificities may have evolved. Whereas GNA plays a role in insect defense, GLIPs seem to be involved in fungal defense.

GLIPs are not homologous to any of the known classes of antifungal proteins and their mode of action remains to be resolved. As opposed to the monomeric GLIPs, related dimeric and tetrameric lectins, including GNA, do not display antifungal activity. Small monomeric GLIPs may penetrate more easily into fungal cell walls, the size exclusion limit for fungal cell walls being around 15–20 kDa (Raikhel *et al.*, 1993). The chitin-binding activity of GLIPs may interfere with the chitinous fraction of the fungal cell wall, but, alternatively, GLIPs may affect the fungal cell membrane. In this respect, it is interesting that a plant defensin inhibited the growth of *Saccharomyces cerevisiae* through insertion into and destabilization of the yeast membrane. The insertion resulted from the interaction between the defensin and mannosylated sphingolipids and/or glycosylphosphatidylinositol-anchored membrane proteins (Thevissen *et al.*, 2000). It is tempting to speculate that GLIPs, through their mannose-binding capacity, interact with mannosylated sphingolipids, resulting in membrane destabilization and ultimately in fungal growth inhibition. That GLIPs can bind to membranes is supported by the observation that comitin, a protein with the mannose-binding motif of GLIPs, binds to the mannose residues of membrane glycoproteins (Jung *et al.*, 1996).

Transgenic tobacco plants that express the entire gastrodianin cDNA(s) accumulated 12-kDa proteins with an antifungal activity similar to that of native preparations. Therefore, the processing of the (monocotyledonous) protein is essentially unaffected when it is produced in a dicotyledonous species. Challenging the transgenic plants with fungi will reveal whether or not gastrodianins display *in planta* activity in a heterologous system. Wild-type or engineered genes of the gastrodianin-like type may contribute, as such or in combination with other antifungal genes, to achieve enhanced fungal tolerance in monocotyledonous and dicotyledonous crops through genetic engineering. Gastrodianins may be particularly suited to engineer resistance towards their natural target, *Armillaria mellea*, a fungal species that includes devastating pathogens of many tree species.

Experimental procedures

Plant material

Fresh terminal corms of *Gastrodia elata* Bl. F. *flavida* S. Chow were obtained from a plantation at Fengcheng (Liaoning Province, PR China), and stored at –70°C for later use.

Table 1. Primers and adaptor

cDNA synthesis primer	5′-TTCTAGAATTCAGCGGCCGC$(T)_{30}N_1N_2$–3′
adaptor	5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′ 5′-ACCTGCCC-3′
adaptor-primer 1	5′-CCATCCTAATACGACTCACTATAGGGC-3′
adaptor-primer 2	5′-ACTCACTATAGGGCTCGAGCGGC-3′
primer 1	5′-TCCGAYCGTYTGAACTCNGGNCAYCAR-3′
primer 2	5′-CTCGCCCAGGGYGGCTAYCTRTTYATC-3′
primer 3	5′-GTTACCGTTTTGGCGATTGGTGTTGCT-3′
primer 4	5′-TGCCCATATTGCCCTGCTACCGCTATA-3′
primer 5	5′-GGTATTCCACCTAGCCATCAAGCAGCC-3′
primer 6	5′-ATGGCAGCATCCGCAAGCACTGCGGTA-3′
primer 7	5′-TATTCTCTTAGACCGCTAGTACATGGA-3′
primer 8	5′-ATTGTTAACCCCGGGTCGGACCTGTTAAATTCCGGC C-3′
primer 9	5′-AGGTCGACTCTAGACTAATTTCCAACGTTGGTGTGGG-3′

N_1 = G/A/C; N_2 = G/A/C/T, Y = C/T; N = A/C/G/T; R = A/G.

Peptide sequence analysis

Gastrodianin was purified according to Xu *et al.* (1998). The purified protein was desalted by reversed phase HPLC on a C4-Vydac column (Bauw *et al.*, 1989) and then lyophilized. The NH_2-terminal amino acid sequence of the protein was determined with a 473 A Protein Sequencer (PE-Applied Biosystems, Foster City, CA, USA). Trypsin digests were performed for 2 h at 35°C on protein dissolved in 50 μl of 0.1 M Tris (pH 8.7) in the presence of 1 μg of porcine trypsin. After purification by reversed phase HPLC on a C4-Vydac column (Bauw *et al.*, 1989), the sequence of some of the isolated peptides was determined with the 473 A protein sequencer.

Chemical cleavage was performed in the dark at room temperature for 36 h on purified and desalted gastrodianin dissolved in 80% acetic acid with 50 μl 3-bromo-3-methyl-2-(2-nitrophenyl-mercapto)-3H-indole (1 mg ml^{-1} in neat acetic acid; Sigma, St. Louis, MO, USA). Excess reagent was removed by centrifugation of the sample after the reaction mixtures were diluted with 20 volumes of H_2O. The peptides were purified by reversed phase HPLC and the sequence was determined.

MS analysis

The mass of gastrodianin and the mass and amino acid sequence of peptides were determined by nanospray ESI-MS using the LC-Q Ion Trap MS (Finnigan, San Jose, CA, USA).

Protein gel blot analysis

Leaves of transgenic plants were ground in liquid nitrogen. After 1/3 volume of extraction buffer (1 M Tris–HCl, 0.5 M EDTA, 50 mM phenylmethylsulfonylfluoride, 0.03% leupeptin, pH 8.0) was added, the extract was centrifuged twice (10 min, 15 000 r.p.m.). Glycerol was added to the supernatant to a final concentration of 20%. Total protein concentration was determined by the Bradford assay (BioRad, Hercules, CA, USA).

Thirty-microgram samples of total protein were separated by 10% SDS-PAGE. After the gel was run, it was shaken for 1 h in balance buffer (50 mM boric acid, 0.1% SDS, pH 8.0) and transferred to a polyvinylidene difluoride membrane at 30 V for 2 h in transfer buffer (50 mM boric acid, 50 mM Tris). The membrane was blocked for 1 h in blocking buffer (0.1% skimmed milk in phosphate buffered saline, 0.05% Tween-20), washed three times (phosphate buffered saline with 0.1% Tween-20), incubated with 1/200 antigastrodianin rabbit antiserum in blocking buffer for 1 h, washed as before, incubated with 1/2500 goat antirabbit IgG in blocking buffer for 1 h and washed three times. After incubation for 1–2 min in reaction buffer (0.3 mg ml^{-1} nitro blue tetrazolium, 0.15 mg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8), the reaction was stopped in sterile water. All incubation steps were performed at room temperature.

RNA isolation

Total cellular RNA was isolated (Chomczynski and Sacchi, 1987) from the outer layer of fresh terminal corms. The resulting RNA preparation was enriched for poly$(A)^+$ RNA with Dynabeads Oligo$(dT)_{25}$ (Dynal AS, Oslo, Norway).

cDNA synthesis was performed with the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA). Essentially, purified mRNA was reverse-transcribed with a cDNA synthesis primer (Table 1) followed by second-strand synthesis, the creation of blunt ends and ligation to an adaptor (Table 1). The adaptor-ligated double-stranded cDNA was diluted 250 times in tricine-EDTA buffer and heated for 2 min at 94°C. After cooling on ice for 2 min, it was used as template for PCR amplification.

3′ rapid amplification of cDNA ends (RACE)

To amplify the 3′ end of gastrodianin cDNA(s), the degenerate primers 1 and 2 (Table 1) were designed based on the N-terminal partial amino acid sequence. RACE was performed essentially according to the manufacturer's instructions (Clontech). For the first round of PCR, the 50 μl reaction mixture contained 5 μl of diluted adaptor-ligated cDNA, 10 pmol of primer 1, 10 pmol of adaptor-primer 1 (Table 1), 10 pmol dNTPs, 1x cDNA reaction buffer and 1x Advantage cDNA polymerase mix. Touch-down PCR was performed in a PE GeneAmp System 9600 as follows: at 94°C for 1 min; 5 cycles at 94°C for 30 sec, 72°C for 3 min; 5 cycles at 94°C for 30 sec, 70°C for 3 min and 25 cycles at 94°C for 20 sec, 68°C for 3 min. The primary PCR product was diluted 50-fold in tricine-EDTA buffer and used as template for a second round of amplification with primer 2 and adaptor-primer 2 (Table 1) for 1 min at 94°C; 3 cycles for 30 sec at 94°C, for 3 min at 72°C; 3

cycles for 30 sec at 94°C, for 3 min at 70°C and 15 cycles at 94°C for 20 sec, 68°C for 3 min. The nested PCR product was purified and cloned into pGEM-T (Promega, Madison, WI, USA) for sequencing.

5′ RACE

Based on the nucleotide sequence of the 3′ RACE product, the specific primers 3 and 4 (Table 1) were designed to amplify the 5′ end of gastrodianin cDNA(s). The first round of PCR was performed with primer 3 and adaptor-primer 1. The PCR product was diluted 50-fold for a second round of amplification with adaptor-primer 2 and primer 4. PCR conditions were as described for 3′ RACE.

Generation of full-length cDNA

Based on the nucleotide sequence of the 3′ and 5′ RACE products, 5′ and 3′ gene-specific primers (primers 5, 6 and 7; Table 1) were designed for the amplification of full-length cDNAs of gastrodianin. A first round of PCR was performed with adaptor-primer 1 and primer 5 and a second round with primers 6 and 7. The thermal cycling program was the same as that utilized for 3′ and 5′ RACE.

Cloning steps for production of gastrodianin in E. coli

Sequences that encoded the two different predicted mature gastrodianin isoforms were amplified from cDNA-containing pGem-T plasmids by PCR using the primers 8 and 9 (Table 1). The forward primer introduced mutations that removed an *Eco*RI restriction site and created a *Bsi*YI restriction site in the N-terminal part of the cDNA. The reverse primer introduced a stop codon adjacent to the C-terminal amino acid of the mature protein and contained, in addition, a *Xba*I site. Amplification products (30 cycles at 94°C for 30 sec, at 55°C for 30 sec, at 72°C for 60 sec; 10 min extension at 72°C) were digested with *Bsi*YI, followed by the removal of protruding 3′ ends with T4 DNA polymerase in the presence of excess dNTPs. After digestion with *Xba*I, the fragments were ligated into *Xmn*I/*Xba*I-digested pMAL-p2 vector (New England Biolabs, Beverly, MA, USA). In the resulting recombinant plasmids, the coding sequences for the mature gastrodianin isoforms were fused translationally downstream from the gene (Maina *et al.*, 1988) that encodes a maltose-binding protein (MBP) and is preceded by a signal sequence for periplasmic targeting. The N-terminal amino acid (S) of mature gastrodianin was positioned right after a cleavage site for the specific protease factor Xa (Nagai and Thøgersen, 1984). A stop codon was inserted after the sequence $NVGN_{140}$ (Figure 1), in accordance with MS data that predicted removal of the C-terminal part of the preproprotein downstream residue N_{140}. Production of the fusion proteins was controlled by the *tac* promoter and repressed through the *lacZ* repressor in the absence of isopropyl-β-D-thiogalactopyranoside. After colonies had been introduced into *E. coli* DH5α, those with inserts were detected by blue-white screening. Correct insertion of the fragments was confirmed through DNA sequencing of the insert region.

Preparation of MBP-gastrodianin fusion proteins and cleavage with factor Xa

MBP-gastrodianin fusion proteins were produced according to the manufacturer's instructions (New England Biolabs). Essentially, log-phase cultures of *E. coli* were induced for fusion protein production for 2 h by addition of isopropyl-β-D-thio-galactopyranoside (0.1 mM). After cells were harvested by centrifugation, the secreted MBP and MBP-fusion proteins were isolated from the periplasmic fraction by cold osmotic shock. After affinity chromatography on an amylose resin, the fusion proteins were recovered in elution buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM maltose) and stored at –20°C. Release of gastrodianin from the fusion proteins was achieved by digestion with factor Xa (3%) in elution buffer (supplemented with 2 mM $CaCl_2$) at room temperature for 3 d. Cleavage was more than 90% complete as judged from Coomassie blue-stained polyacrylamide gels. Concentration and buffer exchange of the intact fusion protein and the digested samples was achieved through three cycles of concentration (Vivaspin concentrator; Vivascience, Lincoln, UK) and buffer addition (50 mM NaAc, pH 5).

Construction of plant transformation vectors and transformation of tobacco

Gastrodianin cDNAs were inserted into the multiple cloning site of pAVAT, a derivative of pTHW136 (Plant Genetic Systems N.V., Gent, Belgium). It contains between the T-DNA borders (from right to left border): a multiple cloning site flanked by the 35S promoter (Odell *et al.*, 1985) with an Ω leader sequence (Gallie and Walbot, 1992) and the 3′ *ocs* (De Greve *et al.*, 1982) termination and polyadenylation signals; the *uidA* gene (Jefferson, 1987) under the control of the TR2′ promoter (Velten *et al.*, 1984) and the 3′ *nos* (Depicker *et al.*, 1982) termination and polyadenylation signals; the *nptII* gene (Herrera-Estrella *et al.*, 1983) under control of the *nos* promoter (Depicker *et al.*, 1982) and the 3′ *ocs* termination and polyadenylation signals. The binary vectors were introduced into *Agrobacterium tumefaciens* C58C1Rif^R carrying the helper plasmid pMP90 (Koncz and Schell, 1986). *Nicotiana tabacum* (L.) SR1 transgenic plants were produced essentially according to Horsch *et al.* (1985).

Purification of gastrodianin from transgenic plants

Transgenic tobacco plants were screened for gastrodianin content by means of protein gel blotting. Selected primary transformants were propagated vegetatively *in vitro*, transferred to soil and grown in the greenhouse. Leaves of transgenic plants were harvested approximately two months after transfer to the greenhouse.

The leaves were homogenized in ascorbic acid (1 g l^{-1}) and the homogenate was centrifuged (3000 *g*, 5 min). The supernatant was adjusted to pH 2.8 with 1 N HCl, centrifuged (3000 *g*, 5 min) and filtered through filter paper. Gastrodianin was subsequently purified by ion exchange chromatography and affinity chromatography on a mannose-Sepharose column. The extract (pH 2.8) was loaded on an 100-ml bed volume column of S Fast Flow (Amersham Pharmacia Biotech, Little Chalfont, UK) equilibrated with 20 mM HAc; the column was washed with 20 mM HAc and proteins were eluted with 0.5 M NaCl in 0.1 M NaOAc (pH 5.0). The pH of the protein fraction was adjusted to 7 with 1 N NaOH. Solid $(NH_4)_2SO_4$ (150 g l^{-1}) was added and the solution was degassed and loaded on a column of mannose-Sepharose 4B (5 ml bed

volume). The column was washed with $(NH_4)_2SO_4$ (150 g l^{-1}) until A280 fell below zero. The proteins were eluted with water and dialyzed against 20 mM HAc. The dialyzed gastrodianin fraction was loaded on a m-Mono-S column (Amersham Pharmacia Biotech) equilibrated with Na-formate (pH 3.8). The column was eluted with a linear gradient (0.05 M NaCl) and gastrodianin eluted as a single peak. The peak fractions were collected and dialyzed against water overnight. Buffer exchange to 50 mM NaAc (pH 5) was done as described above.

Antifungal activity assays

Valsa ambiens was cultivated on potato dextrose agar (Difco Laboratories, Detroit, MI, USA) at 24°C. For testing fungal growth inhibition, mycelium was inoculated in the middle of a potato dextrose agar plate and grown to a diameter of 3 cm. The proteins to be tested were spotted at a distance of approximately 1 cm from the fungal mycelium. Before spotting on the plate, the concentration of samples was estimated by gel blot analysis and samples were adjusted to 20 µl in 50 mM NaAc (pH 5) buffer. Growth inhibition was assessed after incubation for 24 h at 24°C.

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