REVIEW

Microbial Endoxylanases: Effective Weapons to Breach the Plant Cell-Wall Barrier or, Rather, Triggers of Plant Defense Systems?

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Endo- β -1,4-xylanases (EC 3.2.1.8) are key enzymes in the degradation of xylan, the predominant hemicellulose in the cell walls of plants and the second most abundant polysaccharide on earth. A number of endoxylanases are produced by microbial phytopathogens responsible for severe crop losses. These enzymes are considered to play an important role in phytopathogenesis, as they provide essential means to the attacking organism to break through the plant cell wall. Plants have evolved numerous defense mechanisms to protect themselves against invading pathogens, amongst which are proteinaceous inhibitors of cell wall-degrading enzymes. These defense mechanisms are triggered when a pathogen-derived elicitor is recognized by the plant. In this review, the diverse aspects of endoxylanases in promoting virulence and in eliciting plant defense systems are highlighted. Furthermore, the role of the relatively recently discovered cereal endoxylanase inhibitor families TAXI (*Triticum aestivum* xylanase inhibitor) and XIP (xylanase inhibitor protein) in plant defense is discussed.

Additional keywords: ethylene-inducing xylanase (EIX), glycoside hydrolase (GH), hypersensitive response (HR), plant pathogens, signaling cascade.

Endo- β -1,4-xylanases (EC 3.2.1.8) (hereafter referred to as endoxylanases) are produced mainly by microorganisms but can also be found in plants, marine algae, protozoans, crustaceans, insects, and snails (Sunna and Antranikian 1997). Because of their ability to break down xylan, these enzymes are increasingly used in a number of biotechnological processes, including bread-making (Courtin et al. 1999), gluten–starch separation (Christophersen et al. 1997), improving nutritional properties of animal feed (Bedford and Schulze 1998), and the bleaching of cellulose pulp in paper manufacturing (Beg et al. 2001; Christov et al. 1999). However, not only have the various industrial applications of endoxylanases stimulated research on the biochemical and molecular aspects of this important enzyme of the family of glycosyl hydrolases. Being capable of degrading the plant cell wall, endoxylanases, especially from fungal origin, have also attracted attention because of their potential role in phytopathogenesis. Here, we outline the dual role of endoxylanases in promoting virulence of phytopatho-

genic microorganisms and in the induction of plant defense responses, with a focus on recent progress in these research topics.

Xylan, endoxylanases, and endoxylanase inhibitors.

The substrate of endoxylanases, xylan, is composed of β -1,4-linked D-xylopyranosyl residues. This β -(1,4)D-xylan main chain is commonly modified with various substituents, including α -D-glucuronic or α -4-O-methyl-D-glucuronic acid linked via α -1,2-glycosidic bonds and α -L-arabinofuranose linked via α -1,2 or α -1,3 glycosidic bonds or both. The xylan can also be esterified with acetic acid at position 2 or 3, or both, of xylopyranosyl units and with ferulic or *p*-coumaric acid at position 5 of arabinofuranosyl units (Kulkarni et al. 1999; Subramaniyan and Prema 2002). The degree of substitution and the nature of side-chains vary considerably between plant species and cell types (Ebringerová and Heinze 2000). Depending on the botanical origin, xylans are predominantly deposited in different parts of the plant, e.g., woody tissues, corn cobs, sunflower hulls, seeds, and cereal grains are very rich in xylan. It is typically located in the secondary cell wall, where it constitutes roughly 20% of the content, but in gramineous monocotyledonous plants, comparable relative amounts of xylan are also found in the primary cell wall. The abundant presence of xylan in the latter seems to interchange with pectin, and hence, both polysaccharides may perform similar roles in distinct plant cell-wall types (Carpita 1996). Endoxylanases catalyze the initial breakdown of xylan by hydrolyzing internal β -1,4 bonds in the polymer backbone and therefore play a key role in the degradation of this complex polysaccharide, although several other hydrolytic enzymes are needed for efficient and complete degradation (Shallom and Shoham 2003). The regions that accommodate xylose moieties are known as subsites, which are given a number prefixed with – or + depending on whether they bind the glycone or aglycone region of the substrate, respectively, and by definition, the cleavage takes place between subsites –1 and +1 (Davies et al. 1997). Some endoxylanases are sterically hindered and require synergistic action of accessory enzymes like α -arabinofuranosidases and α -glucuronidases (Saha 2000). Endoxylanases belong mainly to glycoside hydrolase families 10 (GH10) and 11 (GH11), although some forms are also classified into families 5, 7, 8, and 43 (Collins et al. 2005).

GH10 endoxylanases typically have a high molecular weight (≥ 30 kDa) and a low pI (Subramaniyan and Prema 2002). The overall structure of the catalytic domain of these

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enzymes is an eightfold β/α barrel resembling a 'salad bowl,' with the active site located in a cleft running across the barrel top. The functional domain may be accompanied by a carbohydrate-binding domain, which is in most cases, however, specific for cellulose (Kulkarni et al. 1999). Two glutamate moieties act as catalytic residues of the enzymatic reaction, which proceeds via a double displacement mechanism (Henrissat and Davies 1997; Rye and Withers 2000). GH10 endoxylanases are generally less selective than GH11 endoxylanases, and unlike the latter, they are able to attack decorated forms of the polysaccharide (Biely et al. 1997). Furthermore, GH10 endoxylanases tend to release shorter oligosaccharides.

GH11 endoxylanases are generally characterized by a low molecular mass (typically around 22 kDa) and a high pI, although endoxylanases exhibiting acidic pI values also are naturally occurring in this family (Törrönen and Rouvinen 1997). Their structure adopts a β -jelly roll fold, which has been likened to a right hand with a two- β strand 'thumb' forming a lid over the active site located in the 'palm' (Törrönen et al. 1994). In consonance with GH10 endoxylanases, two glutamates are implicated in the enzymatic hydrolysis, with retention of anomeric configuration via a double-displacement mechanism. However, GH11 endoxylanases are usually more selective and release larger oligosaccharides, since substituents represent a more serious hindrance to their activity (Biely et al. 1997). Only a few members of GH11 are reported to be modular proteins (Törrönen and Rouvinen 1997).

Like many other carbohydrate-active enzymes, proteinaceous inhibitors of endoxylanases were found to be present in plants. In the late 1990s, their existence was demonstrated for the first time by Debyser and associates (1997), after observing inhibition of endoxylanase activities by wheat-flour protein extracts. Subsequently, proteins representing a first type of endoxylanase inhibitors were purified from wheat flour and were named TAXI (*Triticum aestivum* xylanase inhibitor) (Gebruers et al. 2004). Apart from this work, McLauchlan and associates (1999) discovered a second type of proteinaceous endoxylanase inhibitor in wheat, further referred to as XIP (xylanase inhibitor protein), with inhibition specificities and structural properties different from those of TAXI-type proteins (Juge et al. 2004). TAXI and XIP-type inhibitors have been widely found in cereals (rye, barley, maize, oats, rice, durum, and bread wheat) (Beaugrand et al. 2006; Durand et al. 2005; Goesaert et al. 2004, 2005), and based on sequence data, it can be assumed that they are widespread within the plant kingdom (Durand et al. 2005; Raedschelders et al. 2004). Only recently, a third type of endoxylanase inhibitor belonging to the thaumatin protein family could be identified, which was termed thaumatin-like xylanase inhibitor (Beaugrand et al. 2006). In addition to these endoxylanase inhibitors of plant origin, the existence of a powerful bifunctional alkalo-thermophilic *Bacillus* sp. inhibitor of fungal endoxylanases and aspartic proteinases has been reported (Dash et al. 2001). The potential of endoxylanase inhibitors to affect the functionality of endoxylanases in cereal-based biotechnological processes was recently demonstrated (Frederix et al. 2004; Trogh et al. 2004).

TAXI-type endoxylanase inhibitors are approximately 40-kDa proteins with high pI values (≥ 8.0) (Gebruers et al. 2004) that occur in two molecular forms, A and B. Form A is the full-length protein, while form B is its proteolytically processed counterpart, which consists of two polypeptide chains of approximately 28 to 30 and 10 to 12 kDa that are connected by disulfide bonds. In contrast to XIP, TAXI inhibitors appear to be specific for GH11 endoxylanases from both bacterial and fungal origin but do not affect GH10 endoxylanases (Gebruers et al. 2004). The originally identified TAXI protein was further found to be a mixture of TAXI-I and TAXI-II, which can be

differentiated by their different inhibition activity towards GH11 endoxylanase ExIA from *Aspergillus niger* and their different pI values (Gebruers et al. 2001). Recently, the existence of TAXI-III and TAXI-IV has been reported (Igawa et al. 2005). However, the inhibition specificity of TAXI-III, as determined by these authors, corresponds to that of TAXI-I, while the specificity of TAXI-IV has not yet been reported. Site-directed mutations of Asp37 of ExIA (Tahir et al. 2004) and Asp11 of XynA (Sørensen and Sibbesen 2006) completely abolish TAXI-I-mediated inhibition of these GH11 endoxylanases from *Aspergillus niger* and *Bacillus subtilis*, respectively. Furthermore, the reported crystal structure of TAXI-I in complex with ExIA indicates the importance of the C-terminus for the differences in endoxylanase specificity among different TAXI-type inhibitors (Sansen et al. 2004). This was further explored using site-directed mutagenesis, demonstrating that His374 of TAXI-I stabilizes complex formation (Fierens et al. 2005) and that TAXI-II inhibition specificity bears on the identity of two key residues at positions 294 and 376, which are involved in the interaction at the -2 glycone subsite and the active site of GH11 endoxylanases, respectively (Raedschelders et al. 2005).

XIP-type endoxylanase inhibitors are glycosylated monomeric proteins of approximately 30 kDa with pI values of 8.0 to 9.0 (Durand et al. 2005; Goesaert et al. 2005; Juge et al. 2004). The amino-acid sequence of XIP-I shows overall homology with chitinases belonging to glycosyl hydrolase family 18. However, although XIP-I possesses a triosephosphate isomerase-barrel (β/α)₈-fold and displays structural features typical of this family (Payan et al. 2003), it does not hydrolyze chitin (Elliott et al. 2002; McLauchlan et al. 1999) and, thus, represents a novel class of 'chitinase-like' inhibitors. Out of a range of fungal and bacterial endoxylanases from both GH10 and GH11 families tested for XIP-sensitivity, only those of fungal origin were inhibited by XIP, with the exception of the fungal GH10 *Aspergillus aculeatus* endoxylanase (Flatman et al. 2002; Juge et al. 2004). This apparent specific inhibition of fungal endoxylanases is not due to the binding of XIP-I to glycosylation sites on the fungal enzymes, since both the native and *Escherichia coli*-expressed recombinant forms of *Penicillium funiculosum* endoxylanase are inhibited to the same extent (Brutus et al. 2004). However, on the basis of the crystal structures of XIP-I in complex with *Aspergillus nidulans* (GH10) and *Penicillium funiculosum* (GH11) endoxylanases and the structural determinants of XIP-I specificity derived thereof, Payan and associates (2004) postulated that the inhibitor is able to interact with GH10 and GH11 endoxylanases of both fungal and bacterial origin. Furthermore, these authors showed that XIP-I possesses two independent enzyme-binding sites for GH10 and GH11 endoxylanases and that XIP-mediated inhibition involves substrate-mimetic contacts and interactions occluding the active site. This is in agreement with site-directed mutagenesis studies and the observed competitive mode of inhibition (Tahir et al. 2002, 2004).

Endoxylanases as components of the offensive arsenal of plant pathogens.

It is now well established that phytopathogenic microorganisms secrete a number of hydrolytic enzymes capable of degrading cell-wall polymers to invade the plant tissue and feed on the released nutrients (Walton 1994). Nevertheless, so far only few cell wall-degrading enzymes have been reported to be important pathogenicity factors by demonstrating that their loss had significant impact on the pathogenicity of the microorganism. The majority of these reports refer to pectin-degrading enzymes such as polygalacturonases, pectin methylesterases, and pectate lyases (D'Ovidio et al. 2004). In fact, this is not

too surprising, considering that, for many years, research on cell wall-degrading enzymes has focused on pectic enzymes from phytopathogens of dicotyledonous plants. However, unlike in dicotyledonous plants, pectin is only a minor constituent of graminaceous cell walls, which consist mainly of hemicellulose (Carpita 1996). Since xylans represent a large proportion of the hemicellulosic fraction of cereal cell-wall matrices, xylan-degrading enzymes are expected to be important components of the offensive arsenal of cereal pathogens (Cooper et al. 1988; Wanjiru et al. 2002) and may have a role similar to that of pectic enzymes in infection of dicotyledons. Indeed, during the past 20 years, several indications suggesting that endoxylanases might be pathogenicity factors for phytopathogenic microorganisms have been reported. Traditionally, this indirect evidence has been obtained by analyzing the production of cell wall-degrading enzymes upon infection or by examining the effect of (partially) purified endoxylanase on plant cells and infected tissues. For instance, bacterial strains of *Erwinia chrysanthemi* that infect maize were found to secrete more endoxylanase activity than strains that infect only dicotyledonous plants (Braun and Rodrigues 1993), and fungal pathogens, such as *Septoria nodorum*, that infect graminaceous monocotyledons are reported to secrete more xylan-degrading enzymes than pectinases (Cooper et al. 1988; Lehtinen 1993). *Fusarium*-infected barley was also found to contain considerable amounts of exogenous endoxylanase (Schwarz et al. 2002). A high pI endoxylanase is one of the most abundant enzymes produced by *Stagonospora nodorum* when grown on wheat cell walls, and moreover, in its pure form, it is able to degrade the wheat cell wall (Carlile et al. 2000). Furthermore an endoxylanase from fungal origin was shown to be capable of killing rice cells (Ishii 1988). Additional evidence supporting a role for microbial endoxylanases in pathogenicity has been achieved by cytological and molecular techniques. For example, using tissue-printing and immunostaining analyses, Giesbert and associates (1998) demonstrated that endoxylanases were exclusively present in rye ovaries infected with *Claviceps purpurea*,

strongly suggesting that *Claviceps purpurea* secretes endoxylanase while colonizing its host. During the last decade, the identification and cloning of endoxylanase coding genes (Table 1) has opened perspectives for even more stringent approaches to determine the role of endoxylanases in plant pathogenesis, e.g., by the application of gene-disruption techniques or mRNA expression analyses. Furthermore, the use of molecular genetic techniques has allowed the detailed investigation of individual, recombinant endoxylanases. For instance, heterologous expression and subsequent purification of XynA from a corn strain of *Erwinia chrysanthemi* enabled Hurlbert and Preston (2001) to perform an extensive functional characterization of the enzymatic properties of this endoxylanase. Based on the size of the resulting oligoxyloside products, it was suggested that XynA has a role in loosening the cell-wall structure of the host, thereby allowing other enzymes secreted by the pathogen to gain better access to the host tissue, rather than to participate in a pathway for the catabolism of the hemicellulose (Hurlbert and Preston 2001).

The expression pattern of several endoxylanases from at least seven phytopathogenic microorganisms has been determined. The genes encoding Xyn22, Xyn33, and Xyl-6 of *Magnaporthe grisea* were found to be expressed when the fungus was grown on rice cell walls but not when grown on sucrose (Wu et al. 1995). Subsequent detailed analysis using reverse transcriptase-polymerase chain reaction (RT-PCR) revealed that *xyn22* and *xyn33* transcripts are barely present in infected rice seedlings at 46 h, accumulated to maximum at 84 h, and declined in amount by 96 h postinoculation (Wu et al. 2006). On the other hand, transcripts of *xyl-6* were detectable as early as 24 h postinoculation and accumulated to a PCR-saturated level 68 h postinoculation. In addition, *xyl-6* is expressed more strongly than *xyn22* and *xyn33*, both in culture with rice cell wall as a carbon source and in infected rice leaves (Wu et al. 2006). Thus, although expression of endoxylanases from this phytopathogenic fungus is under control of general regulatory mechanisms (e.g., catabolite repression; Kulkarni et al. 1999)

Table 1. Overview of endoxylanase genes cloned from phytopathogenic microorganisms

Gene	Glycosyl hydrolase family	Phytopathogenic microorganism ^a	GenBank accession no.	Reference
<i>xyl1</i>	11	<i>Ascochyta pisi</i>	Z68891	Lübeck et al. 1997
<i>xynBc1</i>	11	<i>Botrytis cinerea</i>	...	Brutus et al. 2005
<i>xyn11A</i>	11		DQ057980	Brito et al. 2006
<i>xyl1</i>	11	<i>Cochliobolus carbonum</i>	L13596	Apel et al. 1993
<i>xyl2</i>	11		U58915	Apel-Birkhold and Walton 1996
<i>xyl3</i>	11		U58916	
<i>xyl4</i>	10		AY622513	Tonukari et al. 2002
<i>xyl2</i>	11	<i>Cochliobolus sativus</i>	AJ004802	Emami and Hack 2001
<i>xyl1</i>	11	<i>Claviceps purpurea</i>	Y16969	Giesbert et al. 1998
<i>xyl2</i>	10		Y16970	
<i>xynA</i>	5	<i>Erwinia chrysanthemi</i>	U41750	Keen et al. 1996
<i>xylA</i>	11	<i>Fusarium graminearum</i>	AY289919	Beliën et al. 2005
<i>xylB</i>	11		AJ863566	
<i>xyl2</i>	10	<i>Fusarium oxysporum</i> f. sp. <i>lycopersici</i>	AF052583	Ruiz-Roldan et al. 1999
<i>xyl3</i>	10		AF052582	
<i>xyl4</i>	11		AF246831	Gómez-Gómez et al. 2002
<i>xyl5</i>	11		AF246830	Gómez-Gómez et al. 2001
<i>htxyl1</i>	11	<i>Helminthosporium turcicum</i>	AJ238895	Degefu et al. 2001
<i>htxyl2</i>	11		AJ548879	Degefu et al. 2004
<i>xyn22</i>	11	<i>Magnaporthe grisea</i>	L37529	Wu et al. 1995
<i>xyn33</i>	10		L37530	
<i>xyl-3</i>	11		AY144350	Wu et al. 2006
<i>xyl-4</i>	11		AY144349	
<i>xyl-5</i>	10		AY144348	
<i>xyl-6</i>	10		AY124591	
<i>xynA</i>	10	<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	AF331922	Rajeshwari et al. 2005
<i>xynB</i>	10		AF331922	

^a Names of species are copied as mentioned in the referred work, and hence, the mixing of anamorph and teleomorph names.

^b No GenBank accession number is available.

as reported for many endoxylanases from nonpathogenic microorganisms, each endoxylanase gene exhibits a unique expression pattern influenced by specific conditions, including the stage of infection. This was also observed for other endoxylanases of phytopathogenic origin. For instance, RT-PCR analysis revealed that *xyl5* and *xyl2* from *Fusarium oxysporum* f. sp. *lycopersici* are expressed exclusively during the initial and final stages of disease, respectively, whereas *xyl3* and *xyl4* are expressed throughout the entire cycle of infection on tomato plants (Gómez-Gómez et al. 2001, 2002; Ruiz-Roldan et al. 1999). Also the endoxylanases of the maize pathogens *Cochliobolus carbonum* and *Helminthosporium turcicum* were found to display a differential expression pattern. Apel-Birkhold and Walton (1996) reported that *xyl1* as well as *xyl3* but not *xyl2* mRNAs from *Cochliobolus carbonum* are present in infected plants. Abundant *htxyl2* transcript was detected at early and late stages of infection by *Helminthosporium turcicum*, whereas no detectable *htxyl1* transcript was found by Northern blot analysis (Degefu et al. 2004). In addition, it could be shown that two endoxylanase genes from *Claviceps purpurea* were expressed during all infection stages in rye, although Giesbert and associates (1998) suggested that detailed analyses such as quantitative PCR might show phase-specific variation in their expression. Western blot analysis revealed that XynB from the rice pathogen *Xanthomonas oryzae* pv. *oryzae* is released into the xylem sap during in planta growth of the bacterium (Rajeshwari et al. 2005). Using semiquantitative RT-PCR, Brutus and associates (2005) demonstrated that *xynBc1* mRNAs of *Botrytis cinerea* accumulate during early stages of infection on tobacco leaf tissue. However, no endoxylanase activity could be detected at the early stages of infection. Also, during infection of bean leaves, significant activity of *Botrytis cinerea* endoxylanase could only be observed from 36 h after inoculation, while aggressive infection had already started by 24 h (von Tiedemann 1997). Recently, quantitative PCR was used to reveal the presence of transcripts of another *Botrytis cinerea* GH11 endoxylanase gene, *xyn11A*, during infection of tomato leaves. Expression of *xyn11A* was detected from the beginning of the infection in the fresh-inoculated leaves, but in contrast to the constitutive expression of *xynBc1* during infection of tobacco (Brutus et al. 2005), the level of *xyn11A* transcripts significantly increased between 24 and 48 h postinoculation (Brito et al. 2006). This expression pattern during the first two days of infection was identical to the one observed by Schulze Gronover and associates (2004) with the same endoxylanase gene in the *Botrytis cinerea*-bean interaction. The latter study additionally demonstrated that the levels of *xyn11A* transcript remained stable between 44 and 68 h postinoculation. It is noteworthy that a close inspection of the amino-acid sequences of XynBc1 and Xyn11A reveals that both endoxylanases are nearly identical proteins differing only at residues 48 (Asp/Ala), 99 (Phe/Ser), 145 (Ile/Val), and 188 (Asp/Asn). Probably, *xynBc1* and *xyn11A* represent the same endoxylanase gene and these substitutions are due to sequence polymorphisms between the *Botrytis cinerea* strains used in the cited studies (B05-10 vs. SAS56).

The differential expression patterns of the endoxylanase genes during pathogenesis suggest their involvement in infection and establishment of disease. However, so far targeted disruption of none of the individual endoxylanase genes from *Cochliobolus carbonum* (Apel et al. 1993; Apel-Birkhold and Walton 1996), *Fusarium oxysporum* (Gómez-Gómez et al. 2001, 2002), *Erwinia chrysanthemi* (Keen et al. 1996), and *Magnaporthe grisea* (Wu et al. 1995) has been reported to dramatically affect pathogenicity. Genetic redundancy or the occurrence of multiple endoxylanases in microorganisms has been put forward as one of the main reasons why the gene dis-

ruption mutants of endoxylanases remain pathogenic (Apel-Birkhold and Walton 1996; Gómez-Gómez et al. 2002). To date, there are no mutants of the above-mentioned phytopathogenic microorganisms in which all of the endoxylanase genes have been disrupted, and therefore, the definitive role of endoxylanases in phytopathogenesis of many of these microorganisms still remains an open question. However, an alternative approach to evaluate the role of multiple endoxylanases in pathogenesis is the disruption of transcriptional regulators or conserved signal transduction components, as these proteins normally affect whole sets of endoxylanase genes. In this context, a *Cochliobolus carbonum* mutant disrupted in *SNF1*, a gene encoding a protein kinase required for expression of catabolite-repressed genes, showed reduced virulence and strongly reduced expression of cell wall-degrading enzymes, including endoxylanases (Tonukari et al. 2000). In screening for genes under control of the *Botrytis cinerea* Gα subunit of a heterotrimeric G protein, Bcg1, a protein required for virulence, two endoxylanase genes were identified (Schulze Gronover et al. 2004). Both genes are expressed in planta in the wild type but not in the *bcg1* mutant or in a mutant for the adenylate cyclase gene *bac*. Furthermore, Ray and associates (2000) demonstrated that mutations in the structural genes of the terminal branch of the type II secretion system (T2S), *xpsD* and *xpsF* (*Xanthomonas* protein secretion genes D and F), reduced virulence of *X. oryzae* pv. *oryzae* and resulted in endoxylanase accumulation in the periplasmic space. In addition, the T2S pathway structural gene *xpsE* was reported to be required for endoxylanase and cellulase secretion and full virulence in *Xanthomonas oryzae* pv. *oryzae* (Sun et al. 2005). Further research revealed that mutation of *xynB*, an endoxylanase gene identified subsequently, partially affects virulence of the pathogen (Rajeshwari et al. 2005). However, severe virulence deficiency required the simultaneous inactivation of another T2S secreted protein, the lipase/esterase LipA, illustrating the functional redundancy among the T2S secreted proteins of *Xanthomonas oryzae* pv. *oryzae* in promoting virulence on rice. It was only recently that experimental evidence demonstrating the requirement of a single endoxylanase for virulence could be obtained. Brito and associates (2006) showed that deletion of *xyn11A* from *Botrytis cinerea* strongly affected its ability to infect tomato leaves and grape berries. The appearance of secondary lesions on tomato leaves was delayed, and the average size for those infections that were actually spreading was reduced by more than 70%. Strikingly, the mutant strain had only a moderate decrease, about 30%, in the level of extracellular endoxylanase activity.

Endoxylanases as elicitors of defense responses in plants.

The interaction between plants and pathogens induces a diverse battery of defense mechanisms, including cell wall strengthening, de novo production of antimicrobial compounds, ethylene biosynthesis, and rapid localized cell death (Atkinson 1993; Greenberg 1997; Morel and Dangel 1997). These defense responses are triggered by pathogen-derived molecules that have been termed elicitors. Proteins exhibiting hydrolytic activity, as well as different cell-wall constituents, originating either from the invading pathogen or host plant have been identified as elicitors (Bucheli et al. 1990; Esquerré-Tugayé et al. 2000). In this context certain endoxylanases are known to be active elicitors (Dean et al. 1989). An endoxylanase from *Trichoderma viride*, referred to as the ethylene-inducing xylanase (EIX), has been used most extensively to study elicitor activity of endoxylanases. When applied to tobacco (Bailey et al. 1990, 1992), pepper, and tomato plants (Avni et al. 1994a), this 22-kDa GH11 endoxylanase induces ethylene production and rapid cell death, symptoms associated with the hypersensitive response (HR). Of major interest in this respect is the fact

that, although HR might provide an effective defense against biotrophic pathogens, it is known to facilitate infection by necrotrophic pathogens (Glazebrook 2005). Regarding this, the relevance of endoxylanases in pathogenesis would particularly hold true for necrotrophic pathogens, and differential expression of endoxylanase genes in hemibiotrophic pathogens may be associated with switching from the biotrophic relationship to the necrotrophic phase.

The tomato cultivar M82 responds to EIX treatment, while a near-isogenic line, cultivar IL75, does not (Avni et al. 1994a). In line with this, also the response of tobacco to EIX was found to be cultivar specific (Bailey et al. 1993). Ron and associates (2000) mapped the EIX-responding locus in tomato by high-resolution linkage analysis. Earlier, Hanania and Avni (1997) had reported the existence of a high affinity-binding site for the EIX elicitor only in membranes of tobacco and tomato that are genetically capable of responding to the elicitor. This, together with the observation that EIX is able to elicit ethylene biosynthesis in protoplasts (lacking most of the cell wall), strongly suggested that the elicitor activity of the endoxylanase is solely based on its specific recognition by a plant protein (Sharon et al. 1993). However, it could not be excluded that the release of cell-wall fragments by the enzymatic xylan-degrading activity takes part in the elicitation process. Two independent studies addressed this question by creating enzymatically inactive forms of XynII from *Trichoderma reesei* (Enkerli et al. 1999) and EIX (Furman-Matarasso et al. 1999) through site-directed mutagenesis of residues in the catalytically active sites. In both studies, enzymatically inactive mutants were obtained that retain elicitor activity, proving that endoxylanase activity is not necessary for elicitor activity. Furthermore, the elicitation epitope on EIX responsible for inducing the hypersensitive-like response has been recently elucidated (Rotblat et al. 2002). Through the generation of EIX-specific polyclonal antibodies and screening of phage-displayed combinatorial peptide libraries an essential sequence of the EIX elicitation activity has been identified. This sequence consists of the pentapeptide TKLGE mapped to an exposed β -strand of EIX. Replacing the pentapeptide TKLGE by VKGT inhibited the elicitation activity but not the enzymatic activity of the EIX protein, further demonstrating that endoxylanase activity is unrelated to the elicitation process (Rotblat et al. 2002). By checking the Pfam database entry of GH11 endoxylanases (accession number PF00457), we only found the TKLGE motif in endoxylanases originating from *Trichoderma* spp. and not from any other saprophytic or pathogenic microbes. In line with this is the intriguing fact that a hypersensitive-like response of plants to endoxylanases from microbes other than *Trichoderma* has so far not been reported.

Recently, two genes, *LeEix1* and *LeEix2*, the products of which act as receptors for EIX, were identified at the EIX-responding locus of tomato (Ron and Avni 2004). Both genes showed homology to the *Ve* and *Cf* resistance genes in tomato, and the encoded proteins contain a Leu zipper, an extracellular Leu-rich repeat domain with glycosylation signals, a transmembrane domain, and a C-terminal domain with a mammalian endocytosis signal. Although both gene products were able to bind the EIX elicitor independently, only *LeEix2* was found to be capable of transmitting the hypersensitive-like response induction signal (Ron and Avni, 2004). Furthermore, diverse signaling cascades have been implicated in response to pathogenic elicitation in plants treated with endoxylanase. Phospholipase C and D, two plant signaling enzymes active in distinctive enzymatic routes for the generation of the lipid second-messenger phosphatidic acid, are activated in response to elicitation of suspension-cultured tomato cells with EIX (van der Luit et al. 2000). Laxalt and associates (2001) demonstrated that expression of

one phospholipase D gene, *LePLD β 1*, is specifically upregulated after endoxylanase treatment. *LePLD β 1*-silenced cell-suspension cultures exhibit a strong decrease in endoxylanase-induced phospholipase D activity and respond to endoxylanase treatment with a disproportionate oxidative burst (Bargmann et al. 2006). In addition, *LePLD β 1*-silenced cells had higher polyphenol oxidase activity, secreted less of the endogenous β -D-xylosidase LeXYL2, and expressed and secreted more xyloglucan-specific endoglucanase inhibitor protein XEGIP. Interestingly, the latter displays sequence similarity with TAXI-type endoxylanase-inhibiting proteins (Raedschelders et al. 2004). Furthermore, a phospholipase D-mediated accumulation of extracellular *N*-acylethanolamines (NAE) was detected in tobacco cell suspensions (Chapman et al. 1998) and leaves (Tripathy et al. 1999) treated with endoxylanase. One of these NAE, *N*-myristoyl ethanolamine (NAE 14:0), activates expression of Phe ammonia lyase-encoding plant defense genes in a manner similar to endoxylanase treatment (Tripathy et al. 1999). Additionally, Tripathy and associates (2003) observed high-affinity specific binding activity for NAE 14:0 in tobacco, supporting the existence of an NAE-signaling pathway that participates in endoxylanase elicitor perception. Another defense signaling pathway that plays a role in the response to elicitation with endoxylanase is the mitogen-activated protein (MAP) kinase cascade. Indeed, the tomato MAP kinase *LeMPK3* is induced at the mRNA level upon treatment with EIX (Mayrose et al. 2004). Further detailed characterization of *LeMPK3* revealed that it exhibits a dual phosphorylation specificity, which prompted Mayrose and associates (2004) to propose that it represents a convergence point for different signaling pathways leading to the activation of tomato defense responses. Apart from this work, the induction of MAP kinase-like activities has been observed in the hypersensitive-like cell death of tobacco cells treated with EIX (Suzuki et al. 1999) and in HR-associated disease resistance (Yang et al. 2001; Yap et al. 2005; Zhang and Liu, 2001). However, despite the involvement of their enzymatic activity in elicitor-signaling, Suzuki and associates (2006) noticed that the expression of MAP kinases, as well as other cell cycle-related genes, in this instance cyclin genes, is reduced upon endoxylanase treatment of tobacco cells. Protein phosphorylation was further shown to be involved in the regulation of endoxylanase-activated GCC box- and W box-mediated transcription of a tobacco class I basic chitinase gene (Yamamoto et al. 1999, 2004). The latter studies additionally provided clear indications for the involvement of various GCC and W box-binding ethylene-responding transcription factors in the endoxylanase-induced transcription of defense genes. In addition to the activation of defense genes, protein kinase activity is required for the repression of cyclin genes in tobacco (Suzuki et al. 2006). The increase in ethylene biosynthesis in planta upon treatment with EIX is also accompanied by the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC), an increase in extractable ACC synthase (Acs) activity, and increases in *Acs* and ACC oxidase transcripts (Avni et al. 1994b). Recently, a *cis* element in the promoter of an ACC synthase gene, *LeAcs2*, from tomato was found to be essential for induction by EIX (Matarasso et al. 2005). Subsequently, a cysteine protease from tomato, *LeCp*, that binds to the *cis* element and activates *LeAcs2* gene expression, was isolated. In tobacco, another class of proteases, namely serine proteases, is involved in the hypersensitive-like response upon EIX-mediated elicitation, but in this case, proteolytic activity was suggested to be responsible for signal transduction (Yano et al. 1999).

Role of endoxylanase inhibitors in plant defense.

To defend themselves against cell wall-degrading enzymes secreted by phytopathogenic microorganisms, plants appear to have evolved proteinaceous inhibitors of these enzymes. Such

inhibitors may limit pathogen invasion either by just reducing the hydrolytic activity of the cell wall-degrading enzymes or by ensuring the generation of elicitor-active polysaccharide fragments that might have been extensively degraded in the absence of the inhibitors (Esquerré-Tugayé et al. 2000). The inhibitors that are studied in most detail in this context are the polygalacturonase-inhibiting proteins, which are found in a variety of dicotyledonous plants and pectin-rich monocotyledonous plants (De Lorenzo et al. 2001). Recently, direct evidence of the involvement of this type of inhibitor in plant defense has been reported (De Lorenzo and Ferrari, 2002; D'Ovidio et al. 2004).

Also, the relatively large amounts of endoxylanase inhibitors in grains (typically 50 to 100 and 200 to 300 ppm for TAXI and XIP-type proteins, respectively) suggests they have a role in plant resistance to pathogen infection. Indeed, several lines of indirect evidence suggest their involvement in plant defense through counteracting endoxylanases secreted by phytopathogenic microorganisms. Firstly, TAXI as well as XIP-type inhibitors are specific for exogenous endoxylanases and exhibit no inhibition activity towards known endogenous cereal endoxylanases (Gebruers et al. 2004; Juge et al. 2004). Therefore, it is unlikely that both types of inhibitors act as regulators of physiological processes such as mobilization of endosperm reserves and remodeling of plant cell walls during growth and development. The intricate recognition specificities of both inhibitors towards diverse endoxylanases from specifically microbial origin might be the result of plant evolution to counteract the many endoxylanases secreted by bacteria and fungi, analogous to polygalacturonase-inhibiting proteins (Payan et al. 2004; Sansen et al. 2004; D'Ovidio et al. 2004). Noteworthy, however, is the fact that XIP-I has the capacity to inhibit plant enzymes (two α -amylase isozymes of barley) in addition to inhibition of exogenous endoxylanases (Sancho et al. 2003). So far, endoxylanases from two phytopathogenic microorganisms have been tested for inhibition by TAXI and XIP. Two GH11 endoxylanases from *Fusarium graminearum* are inhibited by TAXI-I but not by XIP-I (Beliën et al. 2005). The latter was an unexpected result, as XIP had previously been postulated to be typically active against endoxylanases produced by fungi (Juge et al. 2004). In line with the previously reported inhibition specificities of both inhibitors, TAXI-I as well as XIP-I are able to inhibit the GH11 endoxylanase, XynBc1, from *Botrytis cinerea* (Brutus et al. 2005). Strikingly, from the four amino-acid differences that distinguish XynBc1 from Xyn11A, the only endoxylanase to date of which the individual activity has unambiguously been demonstrated to be required for pathogenesis (Brito et al. 2006), two residues are located at XIP-I interaction positions (Ala⁴⁸ and Val¹⁴⁵). Furthermore, no inhibition of XynBc1 by TAXI-II was observed (Brutus et al. 2005). Taken together, these findings suggest a coevolutionary arms race, i.e., a dance of adaptation and counter-adaptation, in which plants evolved different classes of inhibitors to counteract the many endoxylanases secreted by phytopathogens while the latter evolved to produce multiple endoxylanases with distinct sensitivities towards endoxylanase inhibitors.

Secondly, both types of inhibitors show sequence homology with proteins that are known to counteract invading pathogens or, at least, are thought to be involved in plant defense. As stated above, XIP shows high sequence and structural homology with Class III chitinases. These enzymes have been classified as pathogenesis-related proteins because of their inducible expression upon infection by pathogens (Yu and Guo 2000). Chitinolytic activities are found to counteract invading pathogens by degrading polysaccharides of their cell wall (Punja and Zhang 1993). This direct antifungal activity is accompa-

nied by an indirect defense action through releasing oligosaccharide elicitors from fungal chitin (Sahai and Manocha 1993). Plant chitinases evolve rapidly, suggesting that they are critical in the coevolution of plants and pathogens (Bishop et al. 2000). It is thus likely that XIP proteins have evolved from chitinases whose synthesis was already triggered by fungal attack, but instead of interacting with chitin, this new class of proteins developed endoxylanase inhibitory activity (Durand et al. 2005). In this way, evolution might have retained the existing signal recognition and expression-regulation pathways.

TAXI shows high protein sequence similarity with extracellular dermal glycoproteins (Fierens et al. 2003). The latter have been considered to play a role in plant defense due to their rapid increase of expression in response to wounding and their localization in epidermis and dermal tissues (Satoh et al. 1992). Noticeably, TAXI proteins were also found to be more abundant in the wheat kernel outer tissues (Gebruers et al. 2002). Furthermore, as mentioned above, TAXI displays sequence similarity with xyloglucan-specific endoglucanase inhibitor protein XEGIP (Raedschelders et al. 2004), which has been ascribed a role in plant defense by inhibiting microbial glucanases of glycosyl hydrolase family 12 (Qin et al. 2003). This is particularly interesting, since GH12 glucanases share a common β -sandwich fold with GH11 endoxylanases. Noteworthy in this context is the recent identification of a TAXI-resembling protein that belongs to the nectar proteins from tobacco and which has been proposed to protect the gynoeceum from microbial invasion, as a potent inhibitor of fungal endoglucanases (Naqvi et al. 2005). Analysis of the TAXI-I structure further indicated that TAXI-type endoxylanase inhibitors have divergently evolved from an aspartic proteinase ancestor (Sansen et al. 2004). Intriguingly, in their study of pathogenesis-related expression of putative cell-death regulator genes, Hückelhoven and associates (2001) observed that an aspartic proteinase gene is upregulated after inoculation with the powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. Since aspartic proteinases are thought to represent proteins that can disintegrate plant cells (Mutlu and Gal 1999), these authors speculate that this enzyme contributes to HR in barley. Hence, it is tempting to believe that the endoxylanase-inhibition function of TAXI emerged from a class of proteins that was already overexpressed during HR. After all, certain endoxylanases are known to cause hypersensitive-like cell death. Besides, several studies supplying evidence for a role of aspartic proteinases in plant defense response have been published (Guevara et al. 2005; Xia et al. 2004).

Thirdly, TAXI, as well as XIP, proteins have been demonstrated to be wound and pathogen inducible. Igawa and associates (2004) reported that *Taxi-I*, *Taxi-III*, and *Taxi-IV* are strongly expressed in wounded wheat leaves. When challenged by fungal phytopathogens *Fusarium graminearum* and *Erysiphe graminis*, the concentrations of *Taxi-III* and *Taxi-IV* transcripts also increased significantly, whereas the increases in *Taxi-I* transcripts in response to these pathogens were rather limited (Igawa et al. 2004). Therefore, *Taxi-I* can be considered a basal preexisting defense gene, while *Taxi-III* and *Taxi-IV* are adaptively evolved pathogen-inducible *Taxi*-family members. In contrast to *Taxi* genes, *Xip-I* was not transcribed following inoculation with *Fusarium graminearum* of flowering spikelets (Igawa et al. 2005). However, *Xip-I* was strongly expressed in leaves by infection with *Erysiphe graminis* and also by wounding. Thus, unlike *Taxi-I*, *Xip-I* is pathogen inducible, and unlike *Taxi-III* and *Taxi-IV*, its expression depends on the type of phytopathogen, the infected tissue, or both. Besides their different induction pattern in different parts of the plant, *Taxi* and *Xip* mRNA levels were also found to vary during diverse developmental conditions (Igawa et al. 2005). A

different spatiotemporal expression of endoxylanase inhibitors was observed earlier in studies based on immunodetection and measurements of endoxylanase inhibitor activity (Elliott et al. 2003; Goesaert et al. 2004). Additionally, Igawa and associates (2005) showed that expression of *Xip-I* but not *Taxi* genes is drastically elevated by treatment with methyl jasmonate, an essential signal involved in the particular defense or stress pathway (Schweizer et al. 1997). Furthermore, a proteomic study revealed the secretion of XIP-like proteins into the extracellular matrix of maize upon treatment with an elicitor preparation of *Fusarium verticillioides* (Chivasa et al. 2005). Taken together, the different expression profiles and interaction specificities displayed by TAXI and XIP might be indicative of distinct roles in plant defense depending on the type of plant, the part of the plant that is affected by the infection, the stage in plant development, and the type of pathogens or the nature of the isozyme secreted by a phytopathogen.

Final remarks.

In conclusion, structural and functional analysis of microbial endoxylanases and their inhibitors indicates that they are components of the offensive arsenal of phytopathogens and defense system of plants, respectively. The wide degree of functional redundancy and recognition specificity of endoxylanases as well as endoxylanase inhibitors maximizes the possibility of escaping inhibition from the pathogen viewpoint and counteracting cell-wall degradation from the plant viewpoint. In summarizing the recent advances in elucidation of inhibitor susceptibilities and perception of endoxylanases as elicitors, it is intriguing how subtle molecular determinants (often only a few amino-acid differences) that dictate recognition of these enzymes by plant inhibitors as well as plant receptors are. These highly specific interaction features are likely to be the result of coevolutionary adaptations characteristic of an arms race. Whether microbial endoxylanases significantly contribute to plant invasion or, rather, cause the activation of effective plant defense systems seems to depend on the outcome of this arms race.

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