Plant Toxins
DOI 10.1007/978-94-007-6728-7_4-1

Plant AB Toxins with Lectin Domains

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Abstract

As part of their defense system different plant species each express a diverse set of defense proteins, among them proteins with lectin domains. The whole group of plant lectins assembles all proteins that have the ability to recognize and bind specific carbohydrate structures. Based on the sequence and the conformation of the carbohydrate recognition domains several lectin families are distinguished. Although many lectins are composed only of carbohydrate binding domains, several lectins are chimeric proteins composed of a lectin domain and another unrelated domain. In some cases this second domain can be considered as a toxin domain. This chapter focuses on different types of plant AB toxins and their physiological importance in the battle against pathogens and predators. Most information is available on type 2 ribosome-inactivating proteins in which an *N*-glycosidase domain is linked through a disulfide bridge to a lectin domain. More recently chimeric proteins consisting of one or more lectin domains and a dirigent domain or aerolysin domain have also been discovered. Although these AB toxins all consist of a lectin domain and a toxin domain, the nature of the toxin and the lectin domain are different resulting in proteins with different carbohydrate binding properties as well as a different mode of action for toxicity.

Keywords

Aerolysin; Defense; Lectin; Ribosome-inactivating protein; Toxin

Introduction

Plant lectins are a group of proteins with a very long history. All these proteins share the ability to recognize and bind specific carbohydrate structures. Lectins are ubiquitous in nature, present in various kinds of organisms, and because of their particular interaction with carbohydrate structures can play a role in different biological processes.

For a long time lectin research focused on lectins being abundant proteins present in seeds and plant storage tissues. Over the past era hundreds of plant lectins have been reported in literature, and many of them have been characterized in some detail with respect to their carbohydrate binding properties and biological activities. More recently, molecular analysis and sequencing of lectins also allowed to get some insight into the molecular evolution of lectins. A careful analysis of the sequences available combined with relevant data from genome and transcriptome analyses shows that all plant lectins known today can be classified in roughly 12 different families, based on the sequence of the lectin polypeptides and the conformation of their carbohydrate recognition domains (CRDs) (Van Damme 2014).

Interestingly, the carbohydrate specificity of lectins is not strictly linked to the three-dimensional structure of the CRD. Although plant lectins can recognize some simple monosaccharides they show a

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DOI 10.1007/978-94-007-6728-7_4-1

much higher affinity toward more complex oligosaccharides or glycan structures. The fact that several of these carbohydrate binding proteins specifically recognize carbohydrate structures that are absent from plant tissues led to the hypothesis that these lectins can play an important role in plant defense against predators, as proven by bioassays in which either the purified lectins or transgenic tissues overexpressing the lectin were administered to pathogens or insects. The fact that lectins can survive in the digestional tract of insects or animals also explains why some lectins can be considered as toxic proteins. The binding of lectins to glycoconjugates along the gastrointestional tract or at the cell surface often will result in local effects but at the same time can also trigger some systemic effects in the tissue (Vasconcelos and Oliveira 2004).

Some lectins have been proven to be toxic proteins. For instance, the bean (*Phaseolus vulgaris*) agglutinin (PHA) is known to be nutritionally toxic to most animals, including insects, rats, ruminants, birds, and also humans (Van Damme et al. 1998). PHA is mitogenic and acts as a potent growth factor for the gut, leading to hyperplastic growth of the cells and increased gut turnover. Binding of the lectin to cell membrane receptors on gut epithelial cells will also provoke changes in nutrient absorption. Since part of the lectin gets internalized into the cell, the lectin can also affect body metabolism, organs, and the immune system depending on the lectin dose, making PHA one of the most toxic lectins known at present. The ingestion of a few raw *Phaseolus* beans is sufficient to cause symptoms such as nausea, vomiting, and diarrhea in humans. Fortunately, the lectin can be deactivated by sufficient boiling of the beans. However, it should be emphasized that not all lectins are as toxic as PHA. Moreover, a lot of lectins are also present in crops and plant tissues that are eaten raw, such as tomato, garlic, banana, etc.

With the advent of genomics and proteomics, a lot of sequence information became available for numerous genes encoding chimeric proteins consisting of one or more lectin domain(s) linked to unrelated domain(s), e.g., a kinase domain, an F-box domain, a chitinase domain, or a toxin domain (Van Damme et al. 2008). Interestingly, these chimerolectins are more widespread in plants than the lectins composed only of CRDs, suggesting that through evolution these lectin domains have been used as building blocks to create new chimeric proteins with multiple domains and with multiple biological activities. This chapter will focus on those chimeric proteins in which a lectin domain was fused to a toxin domain. This class of proteins can be referred to as AB toxins. AB toxins are known for a very long time and are synthesized by a variety of bacteria, pathogens, and plants. The most well-known AB toxins include Cholera toxin, Shiga toxin, Pertussis toxin, and Anthrax and Ricin (Odumosu et al. 2010). Ribosome-inactivating proteins (RIPs) such as ricin are the most studied group of AB toxins in plants, which consist of a toxic A subunit with *N*-glycosidase activity and a ricin-related lectin domain as the B subunit. More recently AB toxins containing lectin domains of the amaranthin family and the family of jacalin-related lectins have also been identified (Fig. 1). This chapter will focus on the molecular structure of the AB toxins from plants, the biological activity of each of the two domains composing the AB toxin, and their physiological role in the plant.

Ribosome-Inactivating Proteins

RIPs are widely distributed in the plant kingdom and have been detected in Angiospermae or flowering plants from at least 14 families. However, RIPs are not ubiquitous in plants as shown by the absence of a RIP domain from the first completed plant genome of *Arabidopsis thaliana* (Shang et al. 2014). The most famous member of RIPs is ricin, a lethal toxin from castor bean (*Ricinus communis*) seeds, which was the first RIP discovered in plants by Peter Hermann Stillmark in 1888. Hitherto, more than 50 RIPs have been purified and characterized from different plants. The expression level of RIPs is highly variable in plant tissues, ranging from traces of protein to hundreds of milligrams per 100 g fresh weight plant material.

DOI 10.1007/978-94-007-6728-7_4-1

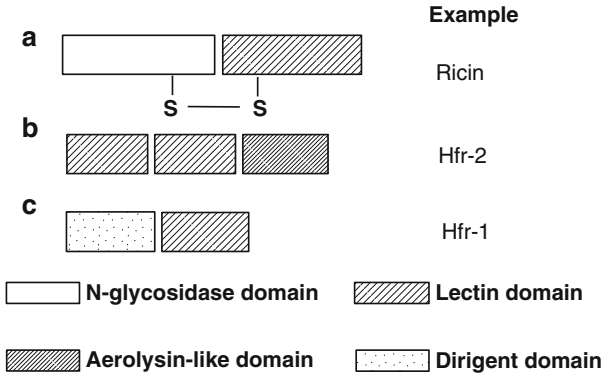

Fig. 1 Overview of the molecular structure of different types of plant AB toxins with lectin domains. (**a**) Schematic structure of type 2 RIP composed on *N*-glycosidase domain and lectin domain linked by a disulfide bridge, (**b**) Amaranthin-like protein with aerolysin domain, (**c**) Jacalin-like protein with dirigent domain

RIPs are not associated with (a) particular tissue(s) but are found in virtually all plant parts (e.g., seeds, roots, leaves, bulbs, fruits, and bark) (Van Damme et al. 2001). The expression of some plant RIP genes is regulated by biotic stresses, such as viral , insect, and fungal infections and by abiotic stresses such as heat and osmotic stress, senescence, salinity, drought, mechanical injury, and oxidative stress. It was reported that transcript levels for RIP genes can also be modulated by plant hormones such as jasmonic acid, abscisic acid, gibberellic acid, and ethylene (Rustgi et al. 2014). For more than a century, the major characteristics of ricin and related proteins have been investigated extensively, including their molecular structures, enzymatic activities, biological roles, and potential applications in agriculture. At present genuine RIPs have been purified mainly from plants but have also been described in bacteria, e.g., Shiga toxin and Shiga-like toxins. It appears, however, that expressed RIP genes occur also in some fungi, algae, as well as a few insects (Shang et al. 2014).

The family of RIPs groups all proteins that possess so-called *N*-glyosidase activity (EC3.2.2.22). These proteins are capable of depurinating a specific adenine residue from what is called the conserved α-sarcin/ricin loop of the large ribosomal RNA (Stirpe and Battelli 2006). The irreversible depurination of the α-sarcin/ricin loop by RIPs renders the ribosomes incapable of binding elongation factor 2, and as a result protein synthesis will be inhibited. Furthermore, RIPs not only depurinate the highly conserved sequence of the α-sarcin/ricin loop within the ribosomal RNA (rRNA) but can also act on naked RNA at multiple sites. It has been demonstrated that some RIPs can remove multiple adenine residues from various polynucleotides (e.g., tobacco mosaic virus RNA), which is referred to as the polynucleotide:adenosine glycosidase activity of RIPs.

Based on their structural organization plant RIPs can be subdivided in three main groups referred to as the type 1, type 2, and type 3 RIPs. Type 1 RIPs consist only of an *N*-glycosidase domain of approximately 30 kDa. Type 2 RIPs are built up of an N-terminal domain with enzymatic activity (A chain) similar to type 1 RIPs, fused to a C-terminal CRD (B chain) of approximately 30 kDa corresponding to the ricin-B lectin domain. Type 2 RIPs are typically described in terms of an AB structure, where the A chain is linked to the B chain through a disulfide bridge (Van Damme et al. 2001). Finally type 3 RIPs consist of an N-terminal RNA *N*-glycosidase domain fused to an unrelated domain with unknown activity. At present a 60 kDa jasmonate-induced protein in barley, referred to as JIP60, is the only protein identified as a type 3 RIP. A recent study reported that the C-terminal domain of JIP60 is similar to the eukaryotic translation initiation factor 4E and plays a role in recruiting a subset of cellular messengers for translation when barley leaves are subjected to jasmonate and senescence stress (Rustgi et al. 2014).

Here the focus will be on the type 2 RIPs, since they are the only type of RIPs with a typical AB structure. Both the A and the B domain are synthesized on one large precursor which undergoes several

DOI 10.1007/978-94-007-6728-7_4-1

processing steps to yield the mature RIP composed of an A domain linked to a B domain by a disulfide bridge (Fig. 1). Since type 2 RIP sequences are generally synthesized with a signal peptide, they follow the secretory route through the endoplasmic reticulum (ER)-Golgi pathway and finally after co- and posttranslational processing of the precursor including the cleavage of the signal peptide and the linker between the A and B domain accumulate in the plant vacuole or the intercellular space.

The overall three-dimensional structure of all type 2 RIPs is very similar to the structure reported for ricin (Robertus and Monzingo 2014). Moreover the amino acid residues important for the catalytic site of the A chain and the sugar-binding sites of the B chain are highly conserved. In contrast to the A chain that is composed of a mixture of α-helices and β-strands, the B chain consists mainly of β-strands. The catalytic site of the A chain contains conserved amino acid residues, which are important for the *N*-glycosidase activity.

The lectin or B chain is a dumbbell-shaped protein consisting of two β-trefoil domains. Each β-trefoil domain is composed of three subdomains (referred to as α, β, and γ) showing a pseudothreefold symmetry, which assembles into a trefoil structure. Cocrystallization of ricin and its complementary carbohydrates revealed that the ricin B chain contains two carbohydrate binding subdomains, corresponding to the α-subdomain of the first β-trefoil domain and the γ-subdomain of the second β-trefoil domain (Robertus and Monzingo 2014). In the case of ricin as well as for most type 2 RIPs the B chain specifically recognizes carbohydrate structures, such as galactose (Gal) and *N*-acetylgalactosamine (GalNAc). As a consequence these RIPs can also be considered as galactose-binding lectins (Van Damme et al. 2001). However, not all RIPs show specificity toward galactose. A few RIPs from *Sambucus* species preferentially interact with sialylated glycans. In particular the type 2 RIP *Sambucus nigra* agglutinin (SNA) I from elderberry bark and homologues of this protein from related elderberry species specifically recognize terminal sialic acid residues (Neu5Ac) α2-6 linked to Gal/GalNAc. Furthermore, the type 2 RIP *Sambucus nigra* lectin–related protein (SNLRP) from elderberry exhibits strong interaction with *N*-acetylglucosamine (GlcNAc) oligomers as well as the $(GlcNAc)_2$ core of *N*-glycans (Shang and Van Damme 2014). The carbohydrate binding domain of IRAb, a type 2 RIP from *Iris* bulbs, has an unusual carbohydrate binding specificity in that it specifically recognizes Gal/GalNAc but also binds mannose (Man) (Hao et al. 2001).

Role of Type 2 RIPs in Plant Defense

As described above RIPs are widely distributed in the plant kingdom but certainly do not occur in all plant species. This suggests that RIPs do not play a universal role in the growth, development, or defense of plants. At present, RIPs have been studied primarily for their toxicity and their unique biological activities.

Several studies demonstrated that RIPs have evolved as plant defense proteins against pathogens or predators, such as fungi, bacteria, viruses, and insects (Stirpe and Battelli 2006). For instance, the highly toxic ricin is responsible to protect *Ricinus communis* seeds from invading pests and pathogens (Barnes et al. 2009). Although most type 2 RIPs show a much lower toxicity for animal cells compared to ricin, the accumulation of these less toxic type 2 RIPs can also play an important role in plant defense (Peumans et al. 2001). A clear distinction should be made between different types of RIPs since only type 2 RIPs can interact with cells and will get into the cytoplasm after binding to suitable glycan receptors on the cell surface and subsequent internalization into the cell. In theory, type 2 RIPs are toxic to all organisms once they gain entry to the cytoplasm of these cells via a receptor-lectin-mediated uptake process. However, the action spectrum of type 2 RIPs is especially directed to animal cells because bacterial and fungal cells are protected by an impenetrable cell wall, which blocks entry of the RIP into the cell (Van Damme et al. 2001). Although type 2 RIPs are sequestered from the host ribosomes in plant cells in, e.g., vacuoles and intercellular spaces, it can be envisaged that once the plant is attacked by pathogens the type 2 RIPs

DOI 10.1007/978-94-007-6728-7_4-1

could enter the plant cytosolic compartments. However, for several type 2 RIPs such as SNA-I, SNA-V, and SNLRP it was reported that they are inactive on plant ribosomes (Vandenbussche et al. 2004a).

Antiviral Activity of Type 2 RIPs

The first discovery of antiviral proteins came from the observation that transmission of tobacco mosaic virus in plants can be inhibited by crude extracts of pokeweed leaves. Afterward, the active protein was isolated and identified as pokeweed antiviral protein, a type 1 RIP from *Phytolacca americana*. Although there is no doubt about the antiviral activity of RIPs, their mode of action has not been elucidated. With respect to the RIP antiviral activity, two major hypotheses have been proposed (Vandenbussche et al. 2004a). (i) The RIPs can directly work on virus nucleic acids by their *N*-glycosidase activity or polynucleotide:adenosine glycosidase activity. Subsequently, the viral protein synthesis is inhibited and the production of virus decreased. (ii) RIPs directly inactivate host ribosomes to limit pathogen spreading by inhibition of translation.

An overview of the data reporting the *in vitro*, *in vivo*, and *in planta* antiviral activities of type 2 RIPs is summarized in Table 1. Ricin, abrin, and modeccin were shown to possess *in vivo* antiviral activity (Stevens et al. 1981). Analyses of the local lesions provoked by the *Eranthis hyemalis* lectin showed *in vivo* antiviral activity of this RIP against alfalfa mosaic virus infection (Kumar et al. 1993). Several type 2 RIPs from *S. nigra* such as SNA-I, SNA-I', SNA-V (or nigrin b), and SNLRP showed the potential to protect transgenic tobacco plants against tobacco mosaic virus infection (Chen et al. 2002; Vandenbussche et al. 2004a). Furthermore, SNA-I, SNA-V, and SNLRP exhibit a potent *N*-glycosidase activity on tobacco mosaic virus RNA by multidepurination of the RNA chain (Tejero et al. 2015). These antiviral activities possibly rely on the direct depurination of the viral genomic RNA, since the expression of SNA-V did not induce the synthesis of pathogenesis-related proteins. Similarly, the type 2 RIP from *Iris* showed antiviral activity to tobacco mosaic virus and tobacco etch virus, without alteration of gene expression for pathogenesis-related proteins (Vandenbussche et al. 2004b; Desmyter et al. 2003).

Table 1 Overview of antiviral activity of type 2 RIPs

RIP	Species and tissue	Antiviral activity[a]			Reference
		In vitro	*In vivo*	*In planta*	
Abrin	*Abrus precatorius* seeds	n.d.[b]	+	n.d.	Stevens et al. 1981
EHL	*Eranthis hyemalis* tubers	n.d.	+	n.d.	Kumar et al. 1993
Modeccin	*Modecca digitata* roots	n.d.	+	n.d.	Stevens et al. 1981
Ricin	*Ricinus communis* seeds	–	+/–	+	Stevens et al. 1981; Taylor et al. 1994
Sambucus nigra agglutinin I (SNA-I)	*Sambucus nigra* bark	+	n.d.	+/– Some lines	Vandenbussche et al. 2004a
SNA-I'		n.d.	n.d.	+	Chen et al. 2002
SNA-V		+	n.d.	+/–	Vandenbussche et al. 2004a
SNLRP		+	n.d.	+/– Some lines	
Iris agglutinin b (IRAb)	*Iris hollandica* bulbs	+	n.d.	+	Vandenbussche et al. 2004b

[a] *In vitro*: PAG activity assay on viral genomic RNA; *In vivo*: bioassay using virus/RIP solution; *In planta*: bioassay using RIP-expressing transgenic plants

[b] n.d.: not determined

DOI 10.1007/978-94-007-6728-7_4-1

Antifungal Activity of Type 2 RIPs

Many fungal ribosomes are highly susceptible to RIPs compared to plant ribosomes (Park et al. 2002; Girbés et al. 2004). There have been many studies describing the antifungal activity of RIPs, particularly for type 1 RIPs. However, RIPs are clearly less potent than other antifungal proteins. For instance, antifungal proteins such as chitinases and thaumatin-like proteins can easily hydrolyze the fungal cell wall or membrane consisting of chitin or β-1,3-glucans, but this is not possible for RIPs. The ricin toxin A chain exhibited enzymatic activity toward the ribosomes from *Rhizoctonia solani* and *Alternaria alternata* in *in vitro* depurination assays (Park et al. 2002). The *Lyophyllum* antifungal protein, a type 2 RIP from *Lyophyllum shimeji* (mushroom), demonstrated enzymatic activity to *Physalospora piricola* in an *in vitro* plate assay, with an IC50 of 70 nM (Lam and Ng 2001). However, this protein did not show any effect to *Rhizoctonia solani*, *Colletotrichum gossypii,* or *Coprinus comatus*. Cinnamomin, the type 2 RIP from the seeds of *Cinnamonum camphora,* has been reported to bind to fungal cells through its B domain and form a cation channel, which allowed the *N*-glycosidase A domain to enter into the cells and resulted in RNA damage (Zhang et al. 1999).

Insecticidal Activity of RIPs

Ricin and saporin were the first RIPs shown to be toxic to insect larvae (Gatehouse et al. 1990). Subsequently, in particular type 2 RIPs received a lot of attention for their insecticidal activities (Vandenborre et al. 2011). An overview of the entomotoxic activity of RIPs is presented in Table 2.

Feeding assays with ricin and cinnamomin revealed the insecticidal activity of type 2 RIPs. Ricin exhibited strong toxicity to several insects including cowpea weevil (*Callosobruchus macultatus*), cotton boll weevil (*Anthonomus grandis*), housefly (*Musca domestica*), and larvae of the silkworm *Bombyx mori* (Wei et al. 2004; Gatehouse et al. 1990). Cinnamomin was toxic, especially toward insect larvae. The LC50 to bollworm (*Helicoverpa armigera*) larvae fed on diet containing cinnamomin was 1839 ppm and the LC50 to mosquito (*Culex pipines pallens*) larvae 168 ppm (Zhou et al. 2000). However, cinnamomin (LD50 is 16599 ppm) was less toxic than ricin (LD50 is 489 ppm) in the feeding assays with the silkworm (*Bombyx mori*) (Table 2).

Since numerous lectins are also toxic to insects (Vandenborre et al. 2011), it is possible that the insecticidal activity of type 2 RIPs should not be attributed to their enzymatic activity but rather could be related to their carbohydrate binding properties. Shahidi-Noghabi et al. (2009) reported that transgenic tobacco plants overexpressing SNA-I or SNA-I' enhanced the resistance to different insect species, including aphids and caterpillars. Mutation of the SNA-I B chain in one carbohydrate binding site reduced the insecticidal activity, while mutation of both carbohydrate binding sites completely abolished the toxic effect. Therefore, the insecticidal properties of the Neu5Acα(2,6)GalNAc/Gal binding SNA-I can be linked to its carbohydrate binding activity (Shahidi-Noghabi et al. 2008).

So far, only few studies investigated the mechanism of RIP toxicity to insects. SNA-I caused cell apoptosis in the gut tissues of *Acyrthosiphon pisum* and *Spodoptera exigua* (Shahidi-Noghabi et al. 2010). Fluorescein isothiocyanate-labeled SNA-I was shown to enter Lepidopteran midgut cells. Furthermore pre-exposure of these midgut cells with specific inhibitors of clathrin- and caveolae-mediated endocytosis inhibited the uptake as well as the caspase-mediated cytotoxicity induced by SNA-I. Though the uptake mechanism(s) required phosphoinositide 3-kinases, it did not depend on the actin cytoskeleton (Shahidi-Noghabi et al. 2011). Recently SNA-I was also shown to be toxic to *T. castaneum* cells as well as larvae, most probably because it is able to cross the peritrophic membrane of the insect gut (Walski et al. 2014).

DOI 10.1007/978-94-007-6728-7_4-1

Table 2 Overview of the entomotoxic activity of type 2 RIPs – updated from Vargas and Carlini 2014

Type 2 RIP	Dose effect	Insect species	Order	Administration	Reference
Ricin from seeds of *Ricinius communis*	LD_{50} 5 × 10^{-4} % (dry wt)	*Callosobruchus maculatus*	Coleoptera	Artificial diet	Gatehouse et al. 1990
	LD_{50} 5 × 10^{-3} % (dry wt)	*Abies grandis*	Coleoptera		
	No effect	*Spodoptera littoralis*	Lepidoptera		
	No effect	*Heliothis virescens*	Lepidoptera		
	LD_{50} 489 mg/kg	*Bombyx mori*	Lepidoptera	Air-dried onto mulberry leaves	Wei et al. 2004
Sambucus nigra agglutinin I (SNA-I) from bark of *Sambucus nigra*	LD_{50} 374 µg/ml	*Acyrthosiphon pisum*	Hemiptera	Artificial diet	Shahidi-Noghabi et al. 2008
	Delayed development and reduced adult survival and fertility	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	
	12 % reduction of larval biomass at 3 days	*Spodoptera exigua*	Lepidoptera	Artificial diet-larvae 5 mg/g SNA-I	Shahidi-Noghabi et al. 2010
	LD_{50} 0.5 µg/ml	*Tribolium castaneum*	Coleoptera	*In vitro* assay with cells	Walski et al. 2014
	20 % mortality feeding diet containing 2 % SNA-I			Artificial diet-larvae	
SNA-I mutant (Asp231ΔGlu) in B chain	Reduced the insecticidal activity of SNA-I	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	Shahidi-Noghabi et al. 2008
SNA-I mutant (Asn48ΔGlu and Asp231ΔGlu) in B chain	Completely abolished the insecticidal activity of SNA-I	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	Shahidi-Noghabi et al. 2008
SNA-I' from bark of *Sambucus nigra*	Reduction of adult aphid survival	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	Shahidi-Noghabi et al. 2009
	Reduction of survival and weight of larvae and pupae	*Spodoptera exigua*	Lepidoptera	Transgenic tobacco	
Cinnamomin from seeds of *Cinnamomum camphora*	LD_{50} 1839 mg/kg	*Helicoverpa armigera*	Lepidoptera	Artificial diet	Zhou et al. 2000
	LD_{50} 168 mg/kg	*Culex pipiens pallens*	Diptera		
	LD_{50} 16,599 mg/kg	*Bombyx mori*	Lepidoptera	Oral feeding	Wei et al. 2004
IRA from bulbs *Iris hollandica*	33 % mortality at 15 days, 100 % mortality at 23 days	*Myzus nicotianae*	Hemiptera	Transgenic tobacco	Shahidi-Noghabi et al. 2006
	31–33 % reduction of adult eclosion	*Spodoptera exigua*	Lepidoptera		

DOI 10.1007/978-94-007-6728-7_4-1

Other Types of AB Toxins in Plants

Sequencing of genomes and transcriptomes of several species together with bioinformatics studies looking for conserved protein domains allowed the identification of chimeric proteins composed of several protein domains, most probably with different biological activities or physiological importance. As already illustrated in the previous section type 2 RIPs are well-studied examples of proteins in which the toxin or *N*-glycosidase domain is linked to a lectin or ricin-B domain. In this section the focus will be on some recently discovered chimeric proteins composed of a toxin domain different from the *N*-glycosidase domain and a lectin domain unrelated to the ricin-B chain.

AB Toxins Composed of Lectin Domain(s) Linked to a Pore-Forming Domain

Several chimeric proteins containing lectin domains fused to an aerolysin domain have been identified in both plants and animals. For instance, in catfish *Plotosus lineatus* two natterin-like toxins (PL-Toxin I and II) were discovered in secretions from skin and venom glands (Tamura et al. 2011). Sequence analysis indicated that these toxins are composed of a jacalin-like lectin domain linked to a toxic aerolysin domain. The family of nattering-like proteins with this AB-type structure has been reported in different fish (Xue et al. 2012). Furthermore, blast searches in databases revealed that sequences encoding chimeric proteins containing Amaranthin-like domain(s) and an aerolysin-like domain are widespread in plants (Liuyi Dang, unpublished data). Amaranthin, a lectin discovered in seeds of *Amaranthus caudatus*, is a 66 kDa nonglycosylated homodimeric protein with two identical carbohydrate binding sites. Structural analysis revealed that each 33 kDa amaranthin subunit contains two homologous domains with a typical β-trefoil fold structure (Transue et al. 1997). Purification of the protein from *Amaranthus caudatus* seeds allowed testing of the biological activity of the protein and showed that Amaranthin exhibits a high specificity for the T-antigen disaccharide (Gal-β1, 3-GalNAc-α-O-) but also interacts with GalNAc. Cloning and sequence analysis revealed that amaranthin is synthesized without a signal peptide, suggesting that the protein is translated from free ribosomes and will reside in the cytoplasmic compartment. At present the occurrence of the family of amaranthin-like lectins is restricted to the family Amaranthaceae (Van Damme et al. 2008). Biological assays with purified proteins as well as transgenic tobacco, potato, and cotton overexpressing amaranthin revealed that the lectin gene can enhance the plants' resistance against aphids (Yang et al. 2011).

Aerolysin belongs to the β-pore-forming toxin superfamily, which are mainly found and characterized from bacteria (Bischofberger et al. 2012). Many pathogens produce these pore-forming toxins to attack the host by forming holes on the cell membrane. Pore-forming toxins usually undergo a conformational change and then assemble into an oligomeric structure, which then promotes a spontaneous membrane insertion (Iacovache et al. 2010). Eventually the disruption of the membrane permeability barrier can lead to cell death (Parker and Feil 2005). Aerolysin is synthesized as a 52 kDa proaerolysin, an inactive precursor with a C-terminal peptide required for the proper folding of the protein into its soluble form. Proteolysis of the loop region that connects the C-terminal peptide with the main body allows the oligomerization of aerolysin into a heptameric ring-like complex that inserts into the target membrane to form the pore. Proaerolysin is an L-shaped molecule with four domains, among which domain 4 contains the C-terminal peptide, domain 3 is responsible for the oligomerization, and an amphipathic loop between domain 3 and 4 generates the 14-stranded β-barrel necessary for the insertion of the protein in the membrane (Degiacomi et al. 2013). Although the aerolysin domain is produced by *Aeromonas* species, aerolysin-like proteins are not restricted to bacteria but are also present in plants and other eukaryotes (Szczesny et al. 2011).

The best-studied protein of this AB type is the Hessian fly responsive-2 protein (Hfr-2), a 55 kDa protein that contains two amaranthin domains linked with an aerolysin domain (Fig. 1). Hfr-2 was

DOI 10.1007/978-94-007-6728-7_4-1

discovered in wheat when changes of gene expression were evaluated during infestation by virulent Hessian fly (*Mayetiola destructor*) larvae. The expression of Hfr-2 was also upregulated following fall armyworm (*Spodoptera frugiperda*) and bird cherry-oat aphid (*Rhopalosiphum padi*) infestations, while little or no changes in transcript levels were observed after wounding, virus infection, and plant hormone treatment like salicylic acid or abscisic acid. Therefore, Hfr-2 is thought to be involved in plant defense against insects or pathogens. Interestingly, because of the presence of pore-forming toxin domain, Hfr-2 may increase membrane permeability and even cause cellular lysis. It has been suggested that Hessian fly larvae may take advantage of this aspect of wheat defense by manipulating Hfr-2 to insert into the plant membrane at the feeding site and obtain water, ions, and other small nutritive molecules from the inner part of cells for larval development (Puthoff et al. 2005).

Jacalin-Like Proteins with Dirigent Domain

The family of jacalin-related lectins is widespread in the plant kingdom and was named after jacalin, a 18 kDa T-antigen binding lectin first discovered in seeds of jackfruit (*Artocarpus integrifolia*). Within the family of jacalin-related lectins two groups of proteins can be distinguished based on their carbohydrate binding properties. The galactose-specific lectins, like jacalin, are synthesized with a signal peptide and mainly reside in the plant vacuole. In contrast the mannose-specific lectins are synthesized without a signal peptide and accumulate in the nucleocytoplasmic compartment of the plant cell. Many mannose-specific jacalin-related domains are linked to a disease response or dirigent domain, thus forming another group of AB-type proteins (Ma 2014). All proteins of this type reported so far have exclusively been found in the Poaceae family. Especially in wheat, almost half of the jacalin-like proteins contain a dirigent domain (Song et al. 2014). It was hypothesized that these chimeric proteins may have evolved from jacalin-related lectins by fusion with a dirigent domain at the N-terminus, which could broaden the physiological role of jacalin-related lectins (Ma 2014).

Dirigent proteins represent a group of proteins which control free radical coupling of monolignol plant phenols, leading to formation of lignans and lignins (Davin and Lewis 2000). They play vital roles in enhancing stress resistance in plants via regulation of lignin and lignan formation and have been found in all land plants studied so far. Dirigent proteins are extracellular glycoproteins, with a molecular weight ranging from 18 to 21 kDa (Pickel and Schaller 2013). Structural analysis of AtDIR6 from *Arabidopsis thaliana* showed that the protein is a homodimer linked with a disulfide bridge and contains β-barrel structures (Pickel et al. 2012).

A typical jacalin-like protein with a dirigent domain from wheat is the 37.5 kDa protein named Hessian fly responsive-1 (Hfr-1) (Fig. 1; Williams et al. 2002). According to glycan microarray analysis the recombinant Hfr-1 shows a strong affinity to Manα1-6(Manα1-3)Man trisaccharide structures. Similar to Hfr-2, Hfr-1 expression is altered after infestation by Hessian fly (*Mayetiola destructor*) larvae, a major dipteran pest of wheat. Hfr-1 expression is also upregulated by abiotic stress such as water-deficit treatments with salicylic acid and benzothiadiazole but not by methyl jasmonate and abscisic acid (Subramanyam et al. 2006). Resistant wheat plants accumulate high levels of Hfr-1 at the larval feeding site, which prevents the avirulent Hessian fly larvae from establishing their feeding sites. Feeding assays with recombinant Hfr-1 revealed an insecticidal activity for Hfr-1 to the dipteran *Drosophila melanogaster*, the cereal aphid *Sitobion avenae*, showing significant detrimental effect on their growth and survival (Subramanyam et al. 2008). It is worthwhile to note that despite the toxicity of Hfr-1 toward cereal aphids the expression of the Hfr 1 gene is not affected by the cereal aphids, which suggested that Hfr-1 may have a general insecticidal activity against wheat pests (Pyati et al. 2012).

Another well-studied group of jacalin-related proteins with a dirigent domain are the jasmonate-regulated proteins from cereals, such as Ta-JA1 (also called JRP-32) from wheat (*Triticum aestivum*). Interestingly, the expression of Ta-JA1 is confined to stem tissues and hardly detectable in leaf and root

DOI 10.1007/978-94-007-6728-7_4-1

tissues. The recombinant Ta-JA1 proteins were able to inhibit the growth of *E. coli*. Overexpression of Ta-JA1 in tobacco plants enhanced the resistance to infection by bacterial (*Pseudomonas syringe* pv tabaci), fungal (*Phytophthora parasitica* var. nicotianae), and viral pathogens (tobacco mosaic virus). Overexpression of the jacalin-related domain alone in tobacco plants conferred the same resistance to *P. syringe* similar to the whole protein while overexpression of the dirigent domain resulted in altered sensitivity of wheat seedlings to salts. It was suggested that the jacalin-related domain of Ta-JA1 provides a basic disease resistance whereas the dirigent domain plays a role in fine-tuning the activity of Ta-JA1 (Ma 2014). In the past few years many jacalin-related lectin genes have been associated with disease resistance, abiotic stress signaling, wounding, insect damage, or multiple stresses (Song et al. 2014). Structural analyses indicated that Hfr-1 and Ta-JA1 share similar three-dimensional structures with other jacalin-related proteins, such as wheat vernalization-related gene 2 (Ver2), wheat chemically induced gene-1(WCI-1), and maize beta-glucosidase-aggregating factor (BGAF) (Ma 2014).

Conclusion and Future Directions

Most plant species contain a large number of different proteins and other compounds to protect themselves against a variety of pathogens and pest insects. During evolution, multiple protein domains have also been combined to create new chimeric proteins, e.g., proteins composed of one or more lectin domains and a toxin domain. Although these AB toxins all consist of a lectin and a toxin domain, the nature of these domains is different for the different types of AB toxins, resulting in proteins with different carbohydrate binding properties as well as a different mode of action for toxicity.

Judging from the three different types of AB toxins with lectin domains known at present in plants it is clear that these AB proteins show different properties and biological activities and probably are likely to complement each other when present in the same plant. Multiple CRDs belonging to different lectin families can recognize different carbohydrate structures. Similarly, the different toxin domains will exert their toxic effects using different modes of action. It is clear that different AB toxins reside in different cell compartments and most probably will also be expressed in different plant tissues.

Though combinations of type 1 and type 2 RIPs within one species have been reported, e.g., in *Iris* and elderberry, there is no evidence for the occurrence of multiple types of AB toxins within one plant species. Eventually, if desirable, transgenic lines expressing multiple AB toxins could be created to check the cooperative activity between AB toxins of different types. The characterization of other combinations between lectin and toxin domains represents a future challenge and can help to elucidate the biological and physiological importance of these proteins for plant growth and defense.

Acknowledgments

This research was supported by the Research Council of Ghent Univeristy and the Fund for Scientific Research (FWO-Vlaanderen, Brussels, Belgium). Chenjing Shang and Liuyi Dang are recipients of China Scholarship Council and receive doctoral cofunding from the Special Research Council of Ghent University.

DOI 10.1007/978-94-007-6728-7_4-1

Cross-References

- ▶ Antimicrobial and Antifungal Plant Toxins
- ▶ Biotechnological Potential of Ribosome Inactivating Proteins (RIPs)
- ▶ Insecticidal Plant Toxins
- ▶ Ribosome-Inactivating Proteins: An Overview
- ▶ Toxic Ribosome-Inactivating Proteins and Lectins

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