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Mechanisms of sugarcane response to herbivory

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Abstract

Deciphering plant-insect interactions at the molecular level is one of the major topics of interest in contemporary plant biology research. In the last few years, various aspects of the plant response to insect damage have been investigated, including the characterization of direct and indirect responses, the regulation of gene expression resulting from insect attack and the signal transduction pathways. Such research has resulted in the proposal of new methods to enhance host resistance to insect pests, including the use of insecticidal genes that can be transferred by genetic engineering into target crops. By integrating the understanding of how plants react to insect damage with the techniques of molecular biology researchers should be able to increase the wide range of methods available for the control of insect pests. The sugarcane transcriptome project (SUCEST) has allowed the identification of several orthologues genes involved in the plant response to insect damage. In this paper we summarize several aspects of the complex interaction between plants and insects and describe the use of *in silico* analysis to provide information about gene expression in different sugarcane tissues in response to insect attack.

INTRODUCTION

Plants have evolved complex defensive strategies to protect themselves against attack by insects and pathogens, but some insects are able to overcome these defense barriers and cause significant economic losses when they attack cultivated crops. In Brazil, insect pests which attack sugarcane include stem borers (Lepidoptera: Pyralidae) and soil pests (Coleoptera: Curculionidae, Elateridae and Scarabaeidae).

During the twentieth century, highly productive sugarcane varieties with enhanced resistance to insect pests were successfully developed in conventional breeding programs, but few modern crop varieties retain the same degree of resistance as exhibited by their wild relatives. In some cases, it is possible that the resistance genes may still be present, but are not being adequately expressed. Genetically engineered insect-resistant crops could make a major contribution to the development of resistant varieties as a large pool of potential insect control genes exists in other species (Gatehouse and Gatehouse, 1998). Any source of genes encoding desirable characteristics such as pest resistance is therefore of significant interest, and a major source of economically important genes is now emerging from the various genome projects.

The Sugarcane Expressed Sequence Tag (SUCEST) transcriptome project has allowed the identification of several orthologues genes in other species that have been shown to present defined roles in plant defense mechanisms against insect pests. However, it remains to be established whether these genes can be used to enhance the resistance of sugarcane to insect damage.

In this paper we present a current review of plant defense mechanisms and identify sugarcane orthologues genes that are potential targets for the management of insect resistance in sugarcane.

MATERIAL AND METHODS

Identification of cluster consensi

Sugarcane EST cluster consensi were initially identified by basic local alignment search tool (BLAST) searches using the plant protein sequences as the query sequences in the SUCEST database. We selected those plant protein sequences involved in defense against insects described in the literature. EST clusters were identified using the BLAST cut-off value of E < 1e-10, except for alpha-amylase/trypsin inhibitor and carboxypeptidase in which the cut-off value was E < 1e-20. Each EST cluster identified was further used as a query sequence in a new BLAST search in the GENEBANK database, with only those ESTs first aligning to selected proteins being considered as putative sequences.

In silico expression analysis

Assuming that synthesis, binding of the cDNA to the vector, and transformation events occur randomly, the quantity of ESTs in a particular cDNA library is probably

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proportional to the mRNAs expression level. The SUCEST database is composed of un-normalized cDNA libraries from different sugarcane tissues, having a defined number of ESTs per library. Quantitative and qualitative expression analyses of the putative defense proteins in different sugarcane tissues was done *in silico* and a correlation made between the number of ESTs of a particular protein and the total number of ESTs of a cDNA library.

RESULTS AND DISCUSSION

Induced defensive responses to pests are probably activated by signals released during the early stages of insect attack. Although little information is available on the mechanisms operating in sugarcane which defend this plant against insect damage, the response of plants to herbivory has been extensively studied in other plant species. It has been recently estimated that more than 500 genes of *Nicotiana attenuata* are related to herbivory, and about one-half of the known genes are involved in plant-pathogen interactions (Hermsmeier *et al*., 2001). This suggests that a significant amount of genes have overlapping functions in both pathogen and insect attack (for a review see Bruxelles and Roberts, 2001), although specific induction of defensive proteins depending on the biological inducer (insect *vs*., pathogen) has been observed in tomato (Fidantsef *et al.*, 1999). These reports are somewhat contradictory and do not support the hypothesis of a strict dichotomy of signaling pathways driven by insects and pathogens, although they do provide clear evidence for reciprocal induced resistance involving certain pathogens and arthropod herbivores. Additionally, they provide several insights into the integration and coordination of induced defenses against multiple pests, suggesting that expression of resistance against some insects may comprise resistance to others (Stout *et al.* 1999). Interestingly, expression of several defensive proteins has been observed in sugarcane (Table I), suggesting that similar defense mechanisms may exist in different species.

Many plant species release volatile compounds in response to herbivore damage. Two elicitors have been identified in the oral secretions of insect herbivores are able to trigger the production of volatile substances (Figure1). In cabbage leaves, a β-glucosidase from *Pieris brassicae* caterpillars elicits the release of volatile compounds (Mattiacci *et al*, 1995) and volatile production in maize is activated by an elicitor present in the regurgitate of *Spodoptera exigua* (Turlings *et al.*, 1993). In both cases, volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] was identified as the major active elicitor in the oral secretion. The same blend of the volatile terpenoids and indole was released either when maize seedlings were damaged by caterpillar feeding and/or after the seedlings were treated with synthesized or natural volicitin (Alborn *et al.*, 1997, Frey *et al.*, 2000). How the emission of volatile organic compounds functions as a defense-activating mechanism is the major difference between the response of plants to pathogens and to herbivores. The type of defense a plant deploys against a particular herbivore will be highly contingent on the ecological circumstances of the plant (Baldwin and Preston, 1999). It is possible to speculate that there might be emission of volatile compounds in sugarcane in response to herbivory since several genes involved in this signaling pathway are expressed in some of the tissues of this plant.

For several decades plant biologists have been studying the biosynthesis and regulation of specific chemicals associated with plant defense against pests and pathogens (Ryan, 1990). There are numerous classes of naturally occurring phytochemicals that are thought to confer resistance to plants against herbivorous insects. These classes include lectins, waxes, phenolics, sugars, alpha-amylase inhibitors and proteinase inhibitors (Broadway, 1995). Analysis of sugarcane-expressed genes involved in secondary metabolism suggests that most of the expressed compounds may be acting as defensive barriers to insect attack (Table I, Figure 2). However, production of some of these compounds imposes a clear metabolic cost to the plant, as indicated by reduced fitness in the absence of predation, which suggests that production of these compounds is a selective response to insect feeding (Baldwin and Preston, 1999).

Systemic signals induced by herbivory and wounding

Wounding results in the rapid accumulation of inhibitors not only in wounded leaf but also in distal unwounded leaves, indicating that a signal (or signals) released from the wound site travels throughout the plant. These signals include pectic fragments derived from the plant cell wall, jasmonic acid, abscisic acid, electrical potentials, intermediates of the octadecanoid pathway (HPOTre, 12-oxo-PDA), an 18-amino acid polypeptide called systemin isolated from tomato plant leaves, and other plant polypeptide signals (Ryan and Pearce, 2001). Systemin regulates the activation of over 20 defensive genes in tomato plants in response to herbivore and pathogen attack, and has been detected in potato, pepper and nightshade. However, the systemin-encoding gene has not been observed in monocotyledons, suggesting that the response mechanism in dicotyledons is more specific. This observation raises the question as to whether systemic signals might be present in sugarcane.

Several plasma membrane proteins are presented as potential wound signal molecule receptors. A plasma membrane β-glucan-elicitor-binding protein of 70 kDa that binds to fungal elicitors has been isolated from soybean (Umemoto *et al*., 1997), but its function in oligosaccharide signal transduction remains to be elucidated. Interestingly, there is a GEBP orthologue in sugarcane with 65% identity with the soybean protein (Table I). However, its expression was restricted to four out of thirteen libraries, including

Table I - Examples of putative systemic or constituve wound response proteins in sugarcane (clusters obtained from Phrap), available to enhance or introduce new traits for insect resistance.

Gene product	Clusters	e-value (similarity)	Organism	Access
Signal receptor				
β-glucan-elicitor-binding protein (GEBP)	SCEQRT1027G02.g	2e-24 (67%)	*Phaseolus vulgaris*	gi/6625560
Signal pathway-associated				
Putative phospholipase A2	SCCCRZ2CO3F05.g	2e-33 (60%)	*Oryza sativa*	gi/4585708
Ca^{2+} dependent protein kinase	SCACLR2014D07.g	1e-156 (84%)	*Zea mays*	gi/1362190
MAPkinase	SCRLAM1014B03.g	e-100 (88%)	*Arabidopsis thaliana*	gi/2253010
Lipoxygenase (LOX2)	SCCCRT1004E11.g	0.0 (98%)	*Zea mays*	gi/8515851
Allene oxide synthase (AOS)	SCAGLR1043E04.g	(85%)	*Hordeum vulgare*	gi/7452979
Allene oxide cyclase (AOC)	SCJFHR1C08A10.g	6e-64 (69%)	*Lycopersicon esculentum*	gi/8977961
12-oxo-PDA redutase	SCCCCL4013D09.g	e-161 (82%)	*Arabidopsis thaliana*	gi/6707797
Ethylene-response protein (ER)	SCBFST3134E05.g	2e-55 (97%)	*Oryza sativa*	gi/7489538
Ethylene responsive elementbinding factor (EREBP)	SCSGHR1070E03.g	1e-15 (88%)	*Oryza sativa*	gi/9309342
Tryptophan synthase-α	SCCCLR1001A11.g	e-102 (83%)	*Arabidopsis thaliana*	gi/2129755
Indole-3-glycerol phosphate synthase	SCCCLR1080D01.g	e-112 (77%)	*Arabidopsis thaliana*	gi/4587610
Tryptophan decarboxylase	SCBGLR1023E09.g	7e-49 (75%)	*Camptotheca acuminata*	gi/1763279
Defensive-Related Proteins				
Trypsin Inhibitor	SCAGLB2046F01.g	7e-74 (76%)	*Zea mays*	gi/7489819
Cysteine Proteinase Inhibitor	SCEQRT1025E03.g	1e-52 (99%)	*Sorghum bicolor*	gi/1076759
α-amylase Inhibitor	SCACCL6007C04.g	8e-24 (99%)	*Sorghum bicolor*	gi/7451433
α-amylase/trypsin inhibitor (bif.)	SCRLRT3034F06.g	1e-122 (98%)	*Zea mays*	gi/123978
Polyphenol oxidase (PPO)	SCEPAM1051D07.g	0.0 (93%)	*Saccharum sp.*	gi/2737882
Lectins	SCBGST3105H12.g	6e-28 (63%)	*Arabidopsis thaliana*	gi/5903093
Hevein-like protein (HEL)	SCVPRT2073C10.g	1e-59 (91%)	*Zea mays*	gi/2119757
Chitinases	SCJFRT2054F02.g	e-119 (82%)	*Oryza sativa*	gi/9937559
Proteolysis-associated				
Carboxypeptidase	SCBGHR1061B08.g	0.0 (92%)	*Oryza sativa*	gi/100571
Cysteine proteinase (*Mir*1)	SCCCLR1022B11.g	(91%)	*Zea mays*	gi/6682829
Leucine aminopeptidase	SCJFRT2055B04.g	0.0 (78%)	*Lycopersicon esculentum*	gi/7435532

those obtained from plantlets infected with *Glucondatabase diazotroficans* (AD), root (RT) and leaf-root transition zone (RZ) and stem (ST). On the other hand, a wound-inducible systemin cell surface receptor (160 kDa) has been identified in tomato (Scheer and Ryan, 1999) which regulates an intracellular cascade of reactions, including depolarization of the plasma membrane, opening of ion channels, increase of intracellular Ca^{2+}, activation of a mitogen activated protein kinase (MAPkinase) and phospholipase activity (Ryan, 2000). Although systemin has not yet been found in sugarcane, several of its target proteins have been identified (Figure 1, Table I). Another class of plant defense protein is characterized by the presence of extracellular leucine-rich regions (LRRs) that are typical of polypeptide-binding motifs. It has been suggested that many of these proteins might be receptors for polypeptide hormones (Ryan and Pearce, 2001).

Sugarcane presents several ESTs encoding mitogen-activated protein kinases, Ca^{2+} dependent protein kinases and putative phospholipases. These proteins are involved in the activation of the octadecanoid pathway, usually associated with feeding by chewing insects or similar physical trauma. This pathway has been considered as the major route of protein defense activation. The model proposes that wounding and systemin activate a lipase at the cell surface which is responsible for the release of linolenic acid, production of jasmonic acid and the activation of proteinase inhibitor genes (for more details, see Table I and Figure 1).

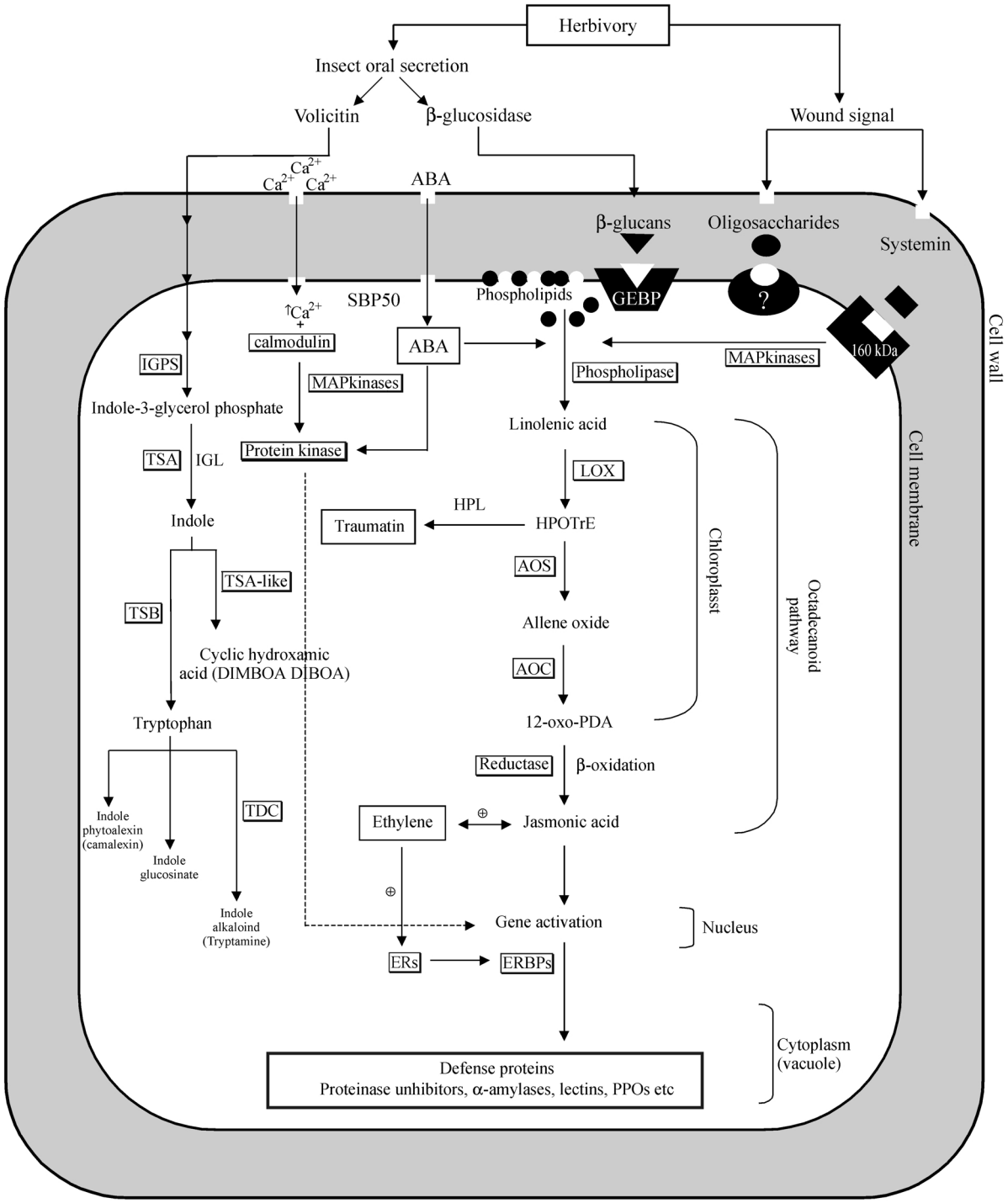

Figure 1 - Putative intracellular wound signal transduction pathway leading to the induction of defense protein gene expression. The pathway is initiated by the interaction of local or systemic signal molecules and putative plasma membrane receptors. Wound signal molecules include oligogalacturonic acid, chitosan, physical signals, abscisic acid (ABA) and systemin. Plasma membrane receptors include a β-glucan-elicitor-binding protein (GEBP), a systemin-binding protein of 160 kDa, and an unidentified receptor for oligosaccharide elicitors. Filled arrows illustrate portions of the pathway proposed from direct evidence. Broken arrows illustrate inferred pathways or interations. Blue boxes indicate orthologous present in sugarcane. A lipase translates the wound signal and releases linolenic acid from membrane phospholipids, a process stimulated by ABA. Volicitin and β-glucosidase from the oral secretion of insects may also function like linolenic acid, i.e. be converted to jasmonic acid through the octadecanoid pathway. **Key:** LOX = lipoxygenase; HPL= hydroperoxyde lyase; AOS = allene oxide synthase; AOC = allene oxide cyclase; SA = salicylic acid; HPOTrE, = 13(S)-hydroperoxylinolenic acid; 12-oxo-PDA = 12-oxo-phytodienoic acid; IGP = indole-3-glycerol phosphate; IGPS = indole-3-glycerol phosphate synthase; TSA = tryptophan synthase-subunit-α (BX1 in maize) (a constitutive enzyme which catalyzes the conversion of IGP to indole); IGL = indole-3-glycerol phosphate lyase (induced in response to herbivore damage in maize); DIMBOA = [2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one] and DIBOA = [2,4-dihydroxy-1,4-benzoxazin-3-one] (defense-related secondary metabolites); TSB = tryptophan synthase-subunit-β; TSA-like = tryptophan synthase-α-like protein (BX2-7 in maize) (enzymes necessary for the conversion of indole to DIMBOA and DIBOA); TDC = tryptophan decarboxylase. ERBPS = ethylene-responsive binding. See text for references.

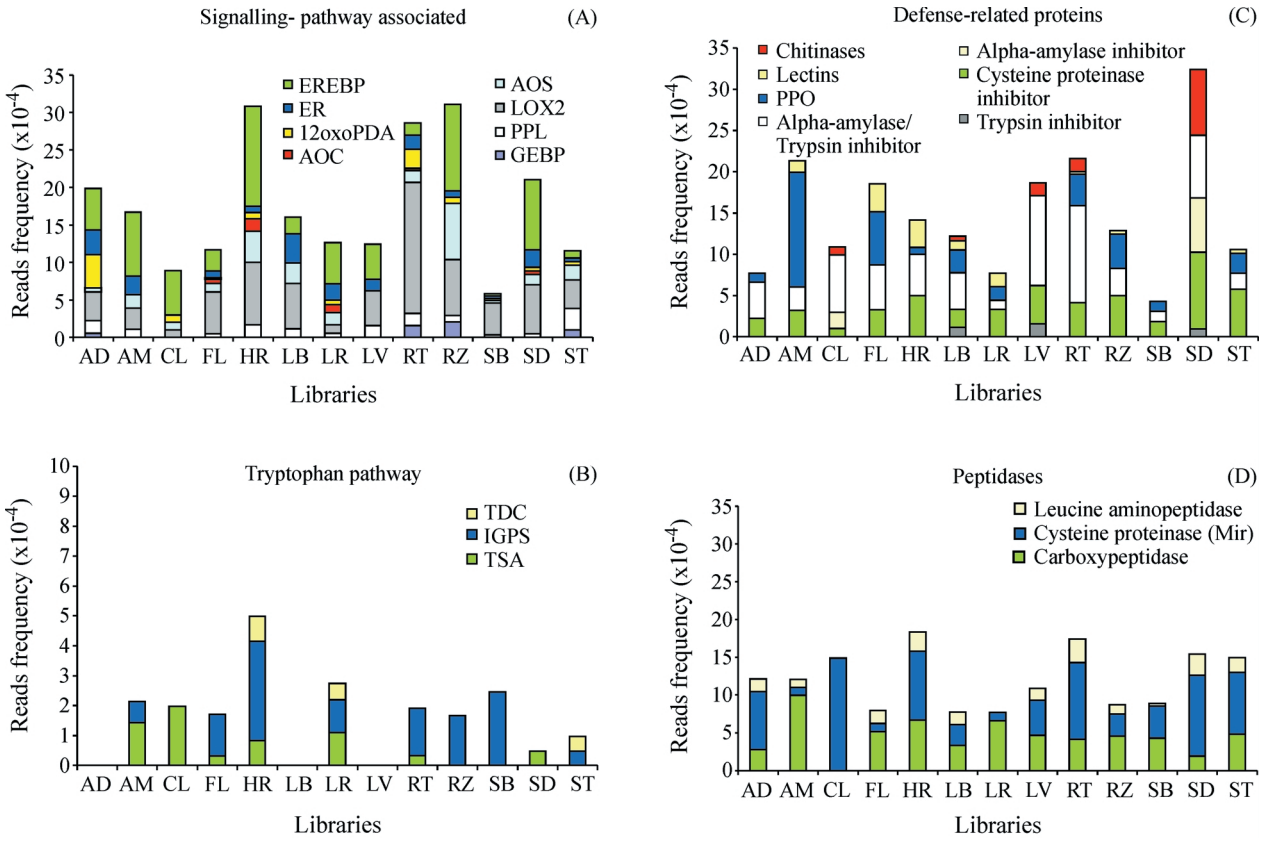

Figure 2 - In silico expression analysis of defense-related proteins against insects using sugarcane cDNA libraries from different tissues. Abbreviations are given in the legend to Figure 1 except when shown. 2A. Signaling-associated pathway (PPL = phospholipase); 2B. Tryptophan pathway; 2C. Defense-related proteins; 2D. Putative peptidases. Abbreviations used in the libraries: **SD**, seeds; **FL**, flowers; **LR**, leaf roll; **LV**, etiolated leaves; **AM**, apical meristem; **LB**, lateral bud; **SB**, stem bark; **ST**, stem; **RZ**, leaf-root transition zone; **RT**, root; **AD**, plantlets without developed leaves and roots infected with *Acetobacter diazotroficans* growing in vitro; **HR**, plantlets without developed leafs and roots infected with *Herbaspirillum rubrisubalbicans* growing *in vitro*; **CL**, calli submited to light/dark and temperature (4 °C and 37 °C) stress.

In the sugarcane octadecanoid pathway, lipoxygenases (LOX) presents high similarity to orthologues in other monocotyledonous, such as maize (98% similarity) and barley (80%). In addition, LOX are expressed in most sugarcane tissues, although in higher amounts only in roots or plants infected with *Herbaspirillum* (Figure 2a). Interestingly, it has been recently shown that *Arabidopsis* lipoxygenases are preferentially activated in response to wounding and dehydration rather than in response to feeding by *Pieris rapae* (Raeymond *et al.*, 2000). Another important protein of the sugarcane octadenoid pathway, allene oxide synthase (AOS), is very similar to the barley orthologues, this enzyme catalyzing the first step in the biosynthesis of jasmonic acid from lipoxygenase-derived hydroperoxides of free fatty acids (Figure 1). Expression of sugarcane AOS appears to be highly regulated, suggesting that it is tissue-specific to the leaf-root transition zone and pathogen-induced (Figure 2a). On the other hand, extremely low mRNA levels for this protein were detected in stem, seeds, flowers and roots (Figure 2a).

The level of abscisic acid (ABA) has been shown to be increased in response to wounding, electrical current, heat treatment or application of systemin. The induction of both ABA and proteinase inhibitor was detected distal to the site of hormone application in an ABA-deficient mutant, showing the mobility of the hormone in plants (Koiwa *et al.*, 1997).

Several molecules have been shown to be potent elicitors of defense proteins in plant cells and many oxylipins have been reported to function as signaling molecules, with the oxylipin traumatin having been suggested as being able to trigger cell division at a wound site, this then leading to the development of a protective callus (Somerville *et al.*, 2000).

Another signaling pathway found in monocotyledonous plants results in the activation of the indole-3-glycerol phosphate lyase (IGL) encoding gene, which is responsible for indole production in maize. IGL catalyses the formation of free indole and is selectively activated by volicitin, the enzymatic properties of IGL being similar to BX1, a maize enzyme that serves as the entry point to the secondary defense metabolites DIBOA and DIMBOA (Figure 1). Gene-sequence analysis indicates that *Igl* (transcriptionally regulated by volicitin) and *Bx1* (constitutively expressed) are evolutionarily related to the tryptophan synthase alpha (TSA) subunit, suggesting that both are derived from TSA

by gene-duplication events and were modified during evolution to obtain their distinct function in secondary metabolism (Frey *et al.*, 2000). This signaling pathway, the tryptophan-associated pathway (TAP) is supposed to occur in sugarcane since most of the orthologues genes have been identified, suggesting a similar response mechanism. TAP ESTs are expressed in most sugarcane tissues, although in very different levels (Figure 2b). For example, TSA is expressed in cDNA libraries from apical meristem, calli, flowers, plantlets infected with *H. rubrisubalbicans,* leaf roll, roots and seeds while indole glycerol phosphate synthase (IGPS), the highly expressed TAP EST, is present in apical meristem, flowers, plantlets infected with *H. rubrisubalbicans,* leaf roll, roots, leaf-root transition zone, stem bark and stem. Tryptophan decarboxylase (TDC), the least expressed EST, has been detected only in plantlets infected with *H. rubrisubalbicans,* leaf-root transition zone and stem (Figure 2b). This enzyme converts tryptophan to the indole-alkaloid tryptamine and can act as an antioviposition and antifeedant agent or as an inhibitor of larval and pupal development (Schuler *et al.*, 1998). Interestingly TDC, IGPS and TSA are expressed in plantlets infected with *H. rubrisubalbicans* but not *Acetobacter diazotroficans* (Figure 2b).

Defense proteins

An interesting strategy against insects is to copy nature by isolating insecticidal genes from non-crop plants and use genetic engineering to transfer them to crop plants thereby enhancing the host's resistance to insects (Boulter, 1993). Screening of world collections has frequently revealed a number of insect resistant biotypes of a particular crop. Protein fractionation and artificial diet bioassays can be used to reveal any protein responsible for at least part of the endogenous resistance (Boulter, 1993).

1. Proteinase Inhibitors

Endopeptidases or proteinases cleave internal peptide bonds in proteins and are separated into four classes, the serine, cysteine, aspartic acid proteinases (classified according to the amino acid side-chain attacked by the enzyme) and the metalloproteinases. Serine proteinases are frequently present as the main digestive enzymes when the pH of the insect midgut lumen content is neutral or alkaline (*e.g.*, Lepidopteran), whereas cysteine and aspartic acid proteinases are frequently found in more acidic gut contents (*e.g.*, Coleopteran) (Terra and Ferreira, 1994). A range of different serine proteinases as well as other types of proteinase enzymes, amylases and lipases have been found to occur in different insects, and it should be possible to screen inhibitor-encoding genes which are effective against specific insects. But not all insects have digestive proteinases, *e.g.* many sucking hemipteran insects depend on free amino acids absorbed from phloem sap as a source of nitrogen nutrients.

Plants have been shown to have inhibitors of all four classes of proteinases. In the SUCEST database there are at least 10 different non-homologous inhibitor families, and multiple isoforms are frequent (Table I). The inhibitors are often found in tissues where one might expect insect attack, *e.g.* seeds, bulbs and leaves. In some cases, the inhibitors are developmentally regulated, while in other cases they are wound-inducible and often lead to a systemic response (Koiwa *et al.*, 1997). Trypsin inhibitors have been detected in leaves, lateral buds, and seed tissue and a bi-functional alpha-amylase-trypsin inhibitor (Figure 2c) in stem, stem bark, apical meristem and leaf roll which are preferential targets for the sugarcane borer *Diatraea saccharalis*. Both types of inhibitors occur in mature leaves and this might indicate an increased level of protection for this particular tissue, since severe damage is usually not observed under field conditions. Cysteine proteinase inhibitors were detected in most sugarcane tissues, although with higher expression in storage organs, such as seeds, stem and leaf-root transition zone (Figure 2c).

Several reports have shown that proteinase inhibitors can delay larval development but do not directly cause mortality (Wolfson and Murdock, 1995). It is assumed that proteinase inhibitors interfere with the digestive process of insects by inhibiting the proteolytic activity of midgut enzymes, thus decreasing the availability of amino acids which in turn leads to depression of the synthesis of proteins necessary for growth, development, and reproduction. In addition, such inhibitors could indirectly affect insect development by a feedback mechanism in which increased production of digestive proteinases (produced to compensate for the low levels of available amino acids) would reduce the pool of amino acids required for the production of essential proteins (Broadway and Duffey, 1986).

The evaluation of proteinaceous inhibitors of proteolytic enzymes as candidates to provide resistance to economically important crops against insect pests is currently attracting much research interest (Ryan, 1990; Jouanin *et al.*, 1998). Several reports have been able to show directly that proteinase inhibitors can inhibit the activity of insect digestive proteinases *in vitro* (for a review, see Jongsma and Bolter, 1997). Soybean proteinase inhibitors incorporated into an artificial diet fed to sugarcane borers (*D. saccharalis*) have produced adverse effects on the growth, development and reproductive potential of this pest (Pompermayer *et al.*, 2001). This observation has allowed us to obtain transgenic plants with increased resistance to the sugarcane borer (manuscript in preparation). However, insects have evolved different strategies to overcome the adverse effects of host proteinase inhibitors (Paulillo *et al.*, 2000, Brito *et al.*, 2001). Our results indicate that bioassays employing proteinase-inhibitor-supplemented diets might be useful in the selection of effective inhibitors for a partic-

ular insect, but validation of feeding trials in transformed plants are also needed.

2. Alpha-amylase Inhibitors

Many of the abundant proteins in cereal seeds are inhibitors of α-amylases, proteinase inhibitors or both. Alpha-amylase inhibitors present in sugarcane are very similar to those found in other monocotyledons such as sorghum and maize (Table I). The amylase inhibitor activities of these proteins are usually directed against alpha-amylases from animals (including insects) and microorganisms, but rarely against amylases from plants. However, considerable variability is found in the specificity of the inhibitors toward alpha-amylases from different species of animals and microorganisms. It thus appears that alpha-amylases inhibitors are part of plants protective chemical mechanisms against pathogens and pests (Ryan, 1990).

Some alpha-amylase inhibitors are similar to inhibitors from some of the families of plant proteinase inhibitors, i.e. the Kunitz, Bowman-Birk and the Ragi/Maize bifunctional inhibitor families (Table I) and some proteinase inhibitors carry reactive sites (located on separated regions) that are able to inhibit alpha-amylases and are considered bi-functional inhibitors (Ryan, 1990).

Wheat alpha-amylase inhibitors are potent inhibitors of the alpha-amylases of several stored-grains insects, including *Tenebrio, Tribolium, Sitophilus* and *Oryzaephilus*. The activity of wheat alpha-amylase inhibitors towards the bean weevil (*Acanthoscelides obtectus*), a commercially important pest, has been addressed by the structural modeling of the enzyme-inhibitor interaction and the determination of the amino acids responsible for the specificity of the inhibitor (Franco *et al.*, 2000).

The insecticidal properties of alpha-amylases have been explored in transgenic plants, with bean alpha-amylase inhibitor 1 expressed in transgenic peas providing complete protection against the pea weevil (*Bruchus pisorum*) under field conditions (Morton *et al.*, 2000). Expression of the transgene had minimal detrimental effect on the nutritional value of peas fed to rats (Pusztai *et al.*, 1999). Analysis of alpha-amylase inhibitor expression in sugarcane has shown that its expression is restricted to callus and seeds (Figure 2c). However, the bi-funtional alpha-amylase/trypsin inhibitor has been detected in most tissues, with significant amounts having been found in seeds, leaves and roots, suggesting that it has a major role as a defense mechanism (Figure 2c).

3. Lectins

Lectins are sugar-binding proteins present in plants, microorganisms and animals and which are especially abundant in the storage organs and protective structures of some plants, particularly legumes. Classification of lectins is based on their sugar-binding properties, and is therefore different to that of the proteinase inhibitors.

The activity of lectins against insects has been investigated in several reports. The common bean (*Phaseolus vulgaris* L.) presents three classes of insecticidal proteins, a lectin (Phaseolus vulgaris agglutinin, PHA) which has been shown to be toxic to the bean weevil *Calosobruchus maculatus*, a related protein, called arcelin, which presents high sequence similarity to PHA and an alpha-amylase inhibitor, all these proteins being products of the same gene family (Gatehouse and Gatehouse, 1998). In addition, Gatehouse *et al.* (1999) evaluated the effects of concanavalin A (ConA), a glucose/mannose-specific lectin from Jacobean (*Canavalia ensiforms*), on two insect crop pests from two different orders, *Lacanobia oleracea* from the Lepidoptera and *Myzus persicae* from the Hemiptera. When expressed in transgenic potato plants, ConA retarded larval development of *L. oleracea*, and decreased larval weights by >45%, while the fecundity of *M. persicae* decreased by up to 45%. Although lectins have been mainly observed in leguminous plants, sugarcane has lectin-like proteins with some similarity to the ones described above. Unlike in leguminous plants, lectin-like gene expression in sugarcane is tissue-specific regulated (Figure 2c), with low expression in sugarcane storage organs and higher expression in flowers and *H. rubrisubalbicans* infected plants. Significant amounts of this protein are also detectable in leaf roll, apical meristem and lateral buds (Figure 2c).

4. Chitinases

Chitin is present in several insect tissues, not only as exoskeletal material, but also in the peritrophic membrane and it is reasonable to assume that chitinases might interfere with insect digestion (Jouanin *et al.*, 1998). Although mainly studied for their anti-fungal properties, chitinases are also interesting as a protective agent against insects, particularly Hemipteran insects (Gatehouse and Gatehouse, 1998). Transgenic potato plants harboring a chitinase encoding gene from bean (bean chitinase, BCH) were found to be resistant to the aphid *Aulacorthum solani* (Gatehouse and Gatehouse, 1998). In addition, expression of an insect chitinase in transgenic plants caused severe damage to the beetle, *Oryzaephilis mercator* (Wang *et al.*, 1996). These data indicate that insect chitinases are a potential resistance factor and might be more potent than chitinases from other organisms (Jouanin *et al.*, 1998). Interestingly, a large set of chitinase has been found in sugarcane where they present regulated expression (Table 1 and Figure 2c), being highly expressed in seeds.

5. Polyphenol oxidases

Polyphenol oxidase (PPO) enzymes are responsible for the typical browning of plant extracts and damaged tissues caused by spontaneous polymerization and cross-linking of *o*-quinones. Fruit commonly contains large amounts of PPO and PPO cDNAs have been cloned from some plant species, including potato, apple, grape and sug-

arcane (Constabel *et al.*, 1995). The physiological function of PPO in fruit and other organs of healthy plants is still uncertain, but a role for leaf PPO in defense against leaf-eating insects has been proposed and documented (Constabel *et al.*, 1995). During chewing and feeding, the mixing of PPO and phenolic substrates generates *o*-quinones and these highly reactive compounds are then able to covalently modify free amino and sulfhydryl groups in dietary proteins within the mouth and gut of the insect. The resulting phenolic adducts prevent efficient assimilation of the alkylated amino acids and thus reduce the nutritive value of protein (Constabel *et al.*, 2000). When PPOs are combined with appropriated phenolic substrates in glandular trichomes, they produce the functional equivalent of `super glue', capable of trapping small-bodied insects. When PPOs are expressed in mesophyll tissues, they can covalently modify and cross-link dietary proteins during feeding, thereby decreasing the proteins' digestibility in the herbivore gut (Baldwin and Preston, 1999). Bellucci *et al.* (1999) found variation of PPO activity in the stems of alfalfa (*Medicago* sp.), with activity being much higher in insect-resistant than susceptible alfalfa, which suggests a possible role for PPO in resistance to biotic stress. PPOs are highly expressed in sugarcane apical meristems (Figure 2c) which is what produces the typical browning of this tissue after cutting or wounding. Additionally, significant mRNA levels also occur in flowers, roots and the root transition zone (Figure 2c).

Multiple insect-resistance genes and their sugarcane orthologues

According to recent data in the literature, hundreds of plant genes are activated after insect feeding (Reymond, 2000) and since plants present a wide variety of defense strategies against insect damage, several genes have to be involved in defense mechanisms against insect feeding.

Bergey *et al.* (1996) studied several systemic wound-response proteins (SWRPs) in transgenic tomato plants in response to overexpression of prosystemin gene. These proteins were classified into various groups: 1. Defense proteins, including proteinase inhibitors (the serine, cysteine and aspartic families) and polyphenol oxidases; 2. Pathway-associated proteins, including pro-systemin, lipoxygenase, calmodulin, nucleotide diphosphate kinase and acyl coA-binding protein; 3. Peptidases, including leucine aminopeptidase, carboxypeptidase, aspartic proteinase, cysteine proteinase and ubiquitin-like protein; and 4. Other proteins. Interestingly, several of these protein-encoding genes are expressed in sugarcane (Table I).

During floral and fruit development, plants utilize over-lapping patterns of expression of the vast array of defense and wound-response genes to ensure production of viable seeds. While the role of protease inhibitors in the control of insect predation is established and highly studied, a few studies have shown that proteases are important in plant defense. For example, leucine aminopeptidases (LAPs) might play a defensive or protective role in tomato flowers and fruit by protecting gametes from insect damage or pathogen attack. Chao *et al.* (1999) suggested that tomato LAP-A can modulate the levels or activities of regulatory proteins or peptides. Therefore, LAP might facilitate the turnover of damaged proteins by reactive oxygen species generated during wounding or it might hydrolyze proteins to supply the pool of amino acids to support the substantial changes in protein synthesis associated with wounding. Expression of LAP in sugarcane is observable in most tissues, except callus and leaf roll, which may suggest an important role for this enzyme in plant defense (Figure 2d).

Another interesting insect-resistance gene from a resistant inbred maize line has recently been isolated and characterized by Pechan *et al.* (2000), the gene product being a 33-kDa cysteine proteinase (encoded by *mir1* gene) which inhibits the growth of a wide range of lepidopteran larvae. Pechan *et al.* suggest that this might be a novel insect defense mechanism in plants, although its mode of action is still unclear. Sugarcane presents at least four different cysteine proteinase encoding cDNAs, and includes a very similar cluster to the maize *mir1* gene and another isoform not yet described in maize (Table I). Its potential application to plant protection against insects is being investigated, particularly against the sugarcane borer, *D. saccharalis*. In agreement with Pechan *et al*. (2000), we have found that *mir1* is highly expressed in sugarcane callus, although higher expression levels were observed in seeds, root transition zone, stem and plants infected with H. *rubrisubalbicans* and A. *diazotroficans* (Figure 2d).

Perspectives

Many studies are in progress to identify new insecticidal products. An endless source of new genes is coming up with the genome, transcriptome and proteome projects, including the sugarcane EST project. Worldwide, various methods are being used to find suitable sources of insect resistance. One strategy is to randomly screen new sources for potential insecticidal proteins, extracts of tropical plants with well-known insecticidal properties being an important possible source. Another strategy is to search plants for known defense proteins which have some evident functional relationship to insect proteins. Designing new genes based on an understanding of protein-protein interactions and phage-display techniques open new frontiers in the development of insect resistant plants. Such projects must be coordinated with the identification of tissue-specific and inducible promoters in target crops and because of this genome projects are essential.

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