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Genetically Modified Soybean for Insect-Pests and Disease Control

Maria Fatima Grossi-de-Sa[1,2], Patrícia B. Pelegrini[1] and Rodrigo R. Fragoso[3]
*[1]Laboratório de Interação Molecular Planta-Praga, Embrapa Recursos Genéticos e
Biotecnologia, Brasília – DF,
[2]Universidade católica de Brasília, Brasília – DF,
[3]Embrapa Cerrados, Planaltina – DF,
Brazil*

1. Introduction

Since 1996, the cultivation of genetically modified (GM) crops around the world has increased more than 80-fold. In 2009, it was registered that a total area of 134 million hectares in 25 countries were used for biotech crops, which constituted a 7 % increase from 2008. 14 million farmers, of whom 90% were small producers, grew GM cultivars in 25 countries during 2009. Nowadays, GM soybean, cotton and corn correspond to 99% of all GM cultivars planted worldwide (JAMES, 2009).

In the same year, with regard to the soybean crop, the planted area reached 90 million hectares worldwide, of which 69 million were GM. The world leaders of soybean production are the United States (33%), Brazil (27%) and Argentina (21%), which are also leaders in the use of GM seeds. The eight following countries also cultivate GM soybean, seven of which are developing countries: Paraguay, South Africa, Uruguay, Bolivia, Mexico, Chile and Costa Rica (JAMES, 2009). Through this use, the GM seed market contributes to an amount of US$ 10.5 billion annually to agriculture. Furthermore, GM soybean, along with corn and cotton, yielded US$ 130 million in 2008 and US$ 143 million in 2009 to the agribusiness sector, with a 10% increase projected for 2010. Each year, countries from the European Union import no less than 40 million tonnes of raw soy products, at a cost of more than U$ 15 billion, mainly from the three biggest producers.

With respect to the agronomical traits used in GM crops, herbicide and insect resistance, or a combination of both traits, are the most utilised. Nevertheless, several other characteristics are being tested, such as increased nutritional quality, dry and cold tolerance, bacteria resistance, fungal and nematode resistances. Therefore, there is an expectation for future development of innovative molecular strategies in order to generate GM plants with novel features that promote reduction of costs and contamination risks for consumers, producers and the environment by decreasing the use of agrotoxic compounds.

Most of the soybean-planted area is comprised of herbicide-tolerant crops (62%), distributed across 11 countries. Transgenic soybean with multiple combined traits corresponds to 21% of all biotech crops around the world (JAMES, 2009). During 2009/2010, a second generation of GM crops appeared in the market. RReady2Yield Soybean was cultivated by 15,000

farmers distributed across 0.5 million hectares in the US and Canada, providing a higher production than traditional soybean crops.

Until January of 2009, there was no GM soybean that was resistant to insect-pests or pathogens being commercialised, although the need for this trait is extremely important. However, an insect-resistant event of GM soybean, developed in China, is in the commercial and regulatory pipeline in that country (STEIN e RODRÍGUEZ-CEREZO, 2009 – Table 1). Today, there is only one commercial GM soybean event from Monsanto, which presents only herbicide-resistance as a trait. Four other GM soybean events are in the commercial pipeline (authorised but not yet commercialised), but all present herbicide resistance genes and demonstrate no resistance against pathogenic fungi, insect-pests or nematodes. In addition, three other soybean events resistant to herbicides are in the regulatory process required for worldwide marketing. Furthermore, two GM soybean events are already at late stages of development, although not yet in the regulatory process. While Monsanto is developing a soybean resistant to insect-pests, Syngenta is leading the development of a cultivar resistant to the cyst nematode (STEIN e RODRÍGUEZ-CEREZO, 2009 – Table 1).

2. Major soybean pests and diseases

Soybean plants originate from the South Asia region, where several microorganisms and insects evolved ecological interactions. All three major soybean-producing countries in the world are located on the American continent, where more than 50% of all soybeans are harvested. This geographic distribution facilitates the spread of insect-pests and diseases. Hence, soybean can be attacked by many different organisms, ranging from viruses to nematodes and insects. These pathogens and pests can cause damage in seeds, roots, leaves, stems and pods, and usually are tissue-specific. Here, we describe some of the most important diseases that attack soybeans, as well as the major pests and pathogens, mainly found in North and South America. A list of pathogens, the diseases they cause and the infected tissues are presented in Table 2.

2.1 Seedling diseases
Diseases that affect soybean seeds occur before germination or after seedling establishment. The primary reason for this is the presence of wet and/or cool soil, which enables the growth of pathogenic fungi, such as *Fusarium* spp, *Rhizoctonia solani*, *Phytophthora sojae* and *Phythium* spp. In some locations, *Macrophomina* spp, *Coletotrichum* spp and *Phomopsis* spp can cause this type of damage in soybean seeds. Seedling diseases can decrease seed quality, due to variation in stands, which are formed by lesions on the cotyledons, soft young stems and primary leaves. Furthermore, these diseases can retard seedling growth, causing moderate to severe losses in crop yield and, in turn, a significant increase in the use of fertilisers and herbicides (MALVICK, 2007).

2.2 Leaf pests and diseases
There are several organisms that attack soybean leaves, including viruses, bacteria, fungi and insects. Among the diseases that have already been described, the most important are soybean rust, septoria brown spot, bacterial blight, bacterial pustule, downy mildew, cercospora leaf blight, frogeye leaf spot, powdery mildew, soybean mosaic virus and bean pod mottle virus.

GM "event" to the market	Developer	Event Name/Genes	Trait	Development stage	Possible commercialisation
Regulatory Pipeline	n/a China	Gna	Insect resistance	Pre-production trials	2010
Advanced R&D Pipeline	Monsanto	n/a	Insect resistance and herbicide tolerance	Phase III	2013
Advanced R&D Pipeline	Syngenta	n/a	Nematode resistance	-	2011
Commercial crop	Monsanto	MON 40-3-2	Herbicide tolerance (to glyphosate)	-	Commercialised
Authorised but not yet commercialised	Monsanto	MON89788	Herbicide tolerance (to glyphosate)	-	2009
Commercial Crop	Bayer CropScience	A2704-12	Herbicide tolerance (to glufosinate)	-	2009
Commercial Crop	Bayer CropScience	A5547-127	Herbicide tolerance (to glufosinate)	-	2012
Authorised but not yet commercialised	Pioneer Hi-Bred	356043	Herbicide tolerance (to ALS inhibitors and glyphosate)	-	2011
Regulatory Pipeline	Pioneer Hi-Bred	305423	Crop composition (High oleic content)	-	2010
Regulatory Pipeline	BASF Plant Science and Embrapa	CV127	Herbicide tolerance (to imidazolinone)	-	2011
Advanced R&D Pipeline	Monsanto	MON87769	Crop composition (Stearidonic acid content)	Phase 3	2012
Advanced R&D Pipeline	Monsanto	n/a	Herbicide tolerance (to dicamba)	Phase 3	2012
Advanced R&D Pipeline	Monsanto	MON87754	Crop composition (high oleic content)	Phase 3	2014
Advanced R&D Pipeline	Dow Agrosciences	n/a	Herbicide tolerance	-	2013
Advanced R&D Pipeline	Syngenta	n/a	Herbicide tolerance (to HPPD inhibitors)	-	2014
Advanced R&D Pipeline	Bayer CropScience	n/a	Herbicide tolerance (to HPPD inhibitors)	Planned 2015	2015
Advanced R&D Pipeline	Bayer CropScience	n/a	Herbicide tolerance (to HPPD inhibitors)	Planned 2015	2015

A=Assessment; (P) = Pending; (E) = Expected; (P) Planned; (T) Tested. Source: Stein and Rodríguez-Cerezo (2009) with modifications

Table 1. GM soybeans from Advanced R&D Pipeline to Commercial Events.

Table 2. Soybean diseases.

Viruses		Bacteria		Fungi	
Diseases	**Species**	**Diseases**	**Species**	**Diseases**	**Species**
Alfalfa mosaic	Alfalfa mosaic virus (AMV)	Bacterial blight	*Pseudomonas amygdale, P. syringae*	Alternaria leaf spot	*Alternaria* spp
Bean pod mottle	Bean pod mottle virus (BPMV)	Bacterial pustules	*Xanthomonas campestris* pv. *glycines*	Anthracnose	*Colleotricum truncatum, C. dematium f. truncatum, C. destructivum, Glomerella glycines, Arkoola nigra*
Bean yellow mosaic	Bean yellow mosaic virus (BYMV)	Bacterial Tan Spot		Black leaf blight	
Brazilian bud blight	Tobacco streak virus (TSV)		*Corynebacterium flaccumfaciens* pv. *flaccumfaciens*	Black root rot	*Thielayopsis basicola, Chalara elegans*
Cowpea chlorotic mottle	Cowpea chlorotic mottle virus (CCMV)		*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	Brown spot	*Septoria glycines, Mycosphaerella usoenskajae*
Mungbean yellow mosaic	Mungbean yellow mosaic virus (MYMV)	Bacterial wilt		Brown stem rot	*Phialophora gregata*
		Wildfire	*Pseudomonas solanacearum*	Charcoal rot	*Macrophomina phaseolina*
Peanut mottle	Peanut mottle virus (PeMoV)		*Pseudonomas syringae* pv. *tabaci*	Choanephora leaf blight	*Choanephora infundibulifera, C. trispora*
Peanut stripe	Peanut Stripe virus (PStV)			Damping-off	*Rhizoctonia solani, Thanatephorus cucumeris, Phythium aphanidermatum, P. debaryanum, P irregular, P. myriotylum, P. ultimum*
Peanut stunt	Peanut Stunt virus (PSV)				
Soybean chlorotic mottle	Soybean chlorotic mottle virus (SbCMV)				
Soybean crinkle leaf	Soybean crinkle leaf virus (SCLV)			Downy mildew	*Peronospora manshurica*
				Drechslera blight	*Dreshslera glycines*
Soybean dwarf	Soybean dwarf virus (SbDV)			Frogeye leaf spot	*Cercospora sojina*
Soybean mosaic	Soybean Mosaic virus (SMV)			Fusarium root rot	*Fusarium* spp
Soybean severe stunt	Soybean Severe Stunt virus (SSSV)			Leptosphaerulina leaf spot	*Leptosphaerulina trifolii*
				Mycoleptodiscus root rot	*Mycoleptodiscus terrestris*
Bud blight	Tobacco ringspot virus (TRSV)			Neocosmospora stem rot	*Neocosmospora vasinfecta, Acremonium* spp
				Phomopsis seed decay	*Phomopsis* spp
				Phytophotora root and stem rot	*Phytophthora sojae*
				Phyllosticta leaf spot	*Phyllosticta sojaecola*
				Phymatotricum root rot	*Phymatotricum omniyorum*
				Pod and stem blight	*Diaporthe phaseolorum, Phomopsis sojae*
				Powdery mildew	*Microsphaera diffusa*
				Purple seed stain	*Cercospora kikuchii*
				Pyrenohcaeta leaf spot	*Pyrenochaeta glycines*
				Pythium rot	*Pythium aphanidermatum, P. debaryanum, P. irregulare, P. myriotylum, P. ultimum*
				Red crown rot	*Cylindrocladium crotalariae, Calonectria crotalariae*
				Read leaf blotch	*Dactuliochaeta glycines, Dactuliophora glycines*
				Rhizoctonia aerial blight	*Rhizoctonia solani, Thanatephorus cucumeris*
				Rhizoctonia root and stem rot	*Rhizoctonia solani*
				Rust	*Pakospora pachyrhizi*
				Scab	*Spaceloma glycines*
				Sclerotinia stem rot	*Sclerotinia sclerotiorum*
				Southern blight	*Sclerotium rolfsii, Athelia rolfsii*
				Steam canker	*Diaporthe phaseolorum, Phomopsis phaseolis*
				Stemphylium leaf blight	*Stemphylium botryosum, Pleospora tarda*
				Sudden death syndrome	*Fusarium solani f.sp. glycines*
				Target spot	*Corynespora casiicola*
				Yeast spot	*Nematospora coryli*

Fungi are the most common soybean pathogens, and include *Phakopsora pachyrhizi* and *P. meibomiae*, which are the main agents of soybean rust, a disease that causes up to 50% of all crop losses in the United States and Mexico. Moreover, *Septoria glycines* is the agent responsible for brown spot, a common disease characterised by defoliation following the formation of brown to red lesions, which affects not only trifoliolate leaves, but also primary leaves and cotyledons (DORRANCE et al., 2008; LUSTER et al., 2010). The fungal-like *Peronospora manshurica* is a worldwide pathogen that causes reduction of seed size and quality due to defoliation, leading to a disease called Downy Mildew (WYLLIE e WILLIAMS, 1965).

When soybean plants contaminated with Soybean Mosaic Virus (SMV) or the Bean pod mottle virus (BPMV), plants become susceptible to other pathogenic agents, especially fungi, which increase damage in the plant and treatment becomes more difficult. Virus diseases can affect seedlings, reducing their quality and germination growth. A decrease in yield is also observed, being less or greater depending on the gravity of the infection (GOLDBACH et al., 1995).

Bacterial infections are widespread diseases that occur mainly in the mid-to-upper and young leaves of the soybean plant. The bacterial blight and bacterial pustules are the most common diseases described thus far. While the first of these infections is characterised by the presence of blight lesions, the second shows light green spots on the surface of the leaves. Both diseases are very similar, rarely causing defoliation or yield loss (WRATHER et al., 2001; YANG, 1997).

Insect-pests are also significant predators of soybean leaves. Some Lepidoptera species, such as *Anticarsia gemmantalis*, known as the velvet caterpillar, cause damage in soybean plants, leading to defoliation (SWAN e PAPP, 1972). *Spodoptera* species also attack the leaves of soybean plants, although they are not tissue-specific and can spread to the pod as well as the entire plant in severe cases. Moreover, some Coleoptera insects have chosen soybean as one of their main target (MUSEUM, 2007). Hence, the Mexican bean beetle (*Epilachna varivestis*) and the Spotted cucumber beetle (*Diabrotica speciosa*) can cause defoliation, decreasing soybean quality and production (CAB/EPPO, 2003; CRANSHAW, 2004). Soybean insect-pests are described in Table 3.

2.3 Stem-and-Pod pests and diseases

Fungi and insects are the major causes of stem and pod damage in soybean. Anthracnose is one of the most well-known fungal diseases, and it attacks soybean plants during wet and warm conditions. *Colleotricum* spp and *Glomerella glycines* may not cause significant yield loss, but they can reduce stand and seed quality (MACHADO e NETO, 2003). Some regions of the U.S. and Canada are commonly attacked by Sclerotinia stem rot (*Sclerotinia sclerotiorum*) every year (PURDY, 1979). In contrast to Anthracnose, these fungi appear during cool weather and resistant varieties of soybean are already being developed.

The insect-pests that target pods and stems in soybean cultivars are varied. Lepidoptera, Coleoptera and Hemiptera species contribute to a decrease in plant production and loss of quality (Table 3). In this way, *Helicoverpa zea* is an insect-pest widely distributed across North and South America that has been recently introduced to the Hawaiian Islands. This pest initially eats soybean leaves but can later attack pods, causing serious yield damage (HARDING, 1976). Furthermore, larvae of the soybean stem borer *Dectes texanus texanus* feed within young soybean plants, resulting in a small yield loss. However, severe stem damages from the inside of the plant can threaten an entire plantation (CAMPBELL e DUYN, 1977).

Order	Soybean tissue attacked	Popular Name	Scientific Name
Lepidoptera	**Leaf**	Green cloverworm Soybean Looper Velvetbean Caterpilar Saltmarsh caterpillar Yellowstriped armyworm	*Plathypena scabra* *Pseudoplusia includens* *Anticarsia gemmatalis* *Estigmene acrea* *Spodoptera ornithogalli*
	Lower leaves to the whole plant	Beet armyworm	*Spodoptera exigua* *Spodoptera latifascia* *Spodoptera eridania*
	Leaf and Pod		
	Pod	Corn Earworm Silverspotted skipper	*Helicoverpa zea* *Epargyreus clarus*
	Stem and Plantlet		*Elasmopalpus lignosellus* *Epinotia aporema*
Coleoptera	Leaf	Mexican Bean Beetle Bean Leaf Beetle Spotted Cucumber Beetle Blister Beetle Japanese Beetle	*Epilachna varivestis* *Cerotoma trifurcata* *Diabrotica undecimpunctata howardi* *Diabrotica speciosa* *Epicauta pestifera, Epicauta lemniscata* *Aracanhtus mourei* *Popillia japonica* *Maecolaspis calcarifera* *Megascelis* sp.
	Stem and Plantlet	Soybean stem borer Grape colaspis Leaf eating beetle	*Dectes texanus texanus* *Colaspis brunnea* *Sternechus subsignatus* *Chalcodermus* sp *Myochrous armatus* *Blapstinus* sp
	Root		*Phyllophaga cuyabana* *Lyogenis suturalis*
Hemiptera	Pod	Green Stink Bug Brown Stink Bug	*Acrosternum hilare, Nezara viridula* *Euschistus servus* *Euchistus heros* *Piezodorus guildinii* *Maruca testulalis* *Etiella zinckenella*
	Root		*Scaptocoris castanea* *Atarsocoris brachiariae* *Dysmicoccus* sp *Pseudococcus* sp
	Stem and Plantlet	Threecornered alfalfa hopper	*Spissistilus festinus* *Dichelops melacanthus e Dichelops furcatus* *Thyanta perditor*
Orthoptera	Leaf	Grasshopper	*Melanoplus* spp

Table 3. Soybean Insect-pests

2.4 Root pests and diseases

Although several fungi and insect species can damage soybean roots, nematodes are the most nocive and well-studied organisms that cause significant yield losses in this crop (Tables 2 and 3). Sedentary phytonematodes are divided into three classes: *Globodera*, *Heterodera* and *Meloidogyne*, and are the most nocive plant pathogens worldwide, causing losses of US$ 125 billion annually in agriculture (CHITWOOD, 2003; SASSER, 1980). Parasitic nematodes are responsible for 12%-20% of all crop losses (KOENNING et al., 1999; SASSER e FRECKMAN, 1987). Worldwide losses caused by nematodes from the *Meloidogyne* genus can reach US$ 16.5 billion per year in some cultivars (TRUDGILL e BLOK, 2001). Among these, *Meloidogyne incognita* is probably the most important nematode for agriculture, due to its worldwide distribution and the wide range of plants that it attacks (EHWAETI et al., 1999; TRUDGILL e BLOK, 2001). In addition, this species is responsible for 95% of nematode infestations around the world. By comparison, species from the genus *Heterodera* cause estimated annual losses of US$ 430 million in the US and US$ 95 million in Europe (MULLER, 1999; SASSER et al., 1983; WRATHER et al., 1997).

The soybean cyst nematode (*Heterodera glycines*) is a widespread pathogen that can be under the soil for years before it can be identified and the host eliminated. It usually causes non-evident symptoms, making diagnosis difficult. This nematode enters the plant root and starts to feed, not visible to the farmers. Consequently, soybean plant losses from this pathogen can reach 30% due to the late diagnosis and difficulties associated with pathogen control (ICHINOHE, 1988).

In addition, root-knot nematodes, represented by many species of *Meloidogyne*, are worldwide-distributed plant-parasites that feed on soybean roots, causing an average of 5% of crop loss around the world (SASSER e CARTER, 1985). They are most common in warm and moist soils, can easily be introduced into soybean roots and are difficult to control (EISENBACK e TRIANTAPHYLLOU, 1991; STIRLING et al., 1992).

In Brazil, the nematode *Pratylenchus brachyurus* can cause losses of 30-50% in some soybean fields. Their life cycle is very short, ranging from 3 to 4 weeks, which enables them to reproduce quickly and present many generations during the same cultivation period. Therefore, the control of *P. brachyurus* is very difficult, once that nematicides and culture rotation are inefficient strategies for the elimination of this plant parasite.

3. Biotechnological strategies to circumvent pests and diseases

3.1 Bt soybean: a future tool on insect-pests control

In nature, there are roughly 100 known bacterial species with potential for insect pathogenesis, but only a few have succeeded as bio-insecticides (ROWE e MARGARITIS, 2004). Among them, formulations based on *Bacillus thuringiensis* (Bt) have showed an effective activity against mosquito larvae and the insecticide activity of this bacteria is due to proteins produced during sporulation, which include a crystal complex (FEDERICI, 2005).

Insects from the Lepidoptera order are particularly susceptible to Cry1 toxins from *B. thuringiensis* (Bt toxins), which are highly toxic after ingestion. They are soluble and are processed by proteases presented in the insect midgut. The active toxins can interact with specific receptors at the midgut, leading to pore formation after toxin conformation change and, consequently, cell death (GILL et al., 1992; KNOWLES, 1994; RAJAMOHAN et al., 1998). Although this is the most commonly suggested method of action for Cry toxins, details about this mechanism, such as pos-binding effects and receptor specificities, are not yet clear (GOMEZ et al., 2002; ZHANG et al., 2005).

In this way, genetic transformation of soybean to induce resistance to lepidopterans using the insertion of Bt toxins has been performed for over a decade. Hence, many techniques have been applied in order to successfully transfer Cry genes into soybean embryos. The bombardment method has provided stable transformed soybean plants (CHRISTOU, 1990; MCCABE e CHRISTOU, 1993). However, the use of *Agrobacterium* for transformation of cotyledonary nodes and the microprojectile bombardment of somatic embryos provided the growth of less fertile transformed plants (FINER e MCMULLEN, 1991; PARROTT et al., 1994; SATO et al., 1993; TOWNSEND e THOMAS, 1993). Nevertheless, years later, it was possible to produce a fertile transformed soybean containing a synthetic *B. thuringiensis* insecticidal crystal protein gene (Cry1Ac) through microprojectile bombardment. These plants showed resistance toward *Helicoverpa zea*, the soybean looper *Pseudoplusia includes*, the tobacco burworm (*Heliothis virescens*) and the velvetbean caterpillar (*Anticarsia gemmatalis*) (STEWART et al., 1996).

In order to increase plant resistance against insect-pests, pyramiding strategies were applied in soybean using a synthetic Bt toxin (Cry1Ac) with native plant resistance genes. Two QTLs from Japanese soybean lines, named 229-H and 229-M, have been described as showing antixenosis and antibiosis resistance towards lepidopteran insects (CREGAN et al., 1999). Hence, a GM soybean presenting a combination of 229-M from the strain PI 22948 and a synthetic *cry1Ac* gene demonstrated resistance against *H. zea, P. includens*, *A. gemmatalis* and *Elasmopalpus lignosellus* (WALKER et al., 2000). Further studies utilised both QTLs, 229-H and 229-M, with the synthetic *cry1Ac* gene for production of transgenic soybean, which showed to be resistant against three lepidopteran insect-pests (*H. zea*, *P. includes* and *H. virescens*) (WALKER et al., 2004). Later, new reports described the production of a GM soybean containing a third QTL, named QTL-G, along with the cry1Ac resistance gene. It was showed that QTL-M presented the largest effect on *P. includens* and *H. zea* resistance. QTL-G worked better against *H. zea* larvae (RECTOR et al., 2000; ZHU et al., 2008), while QTL-H was not as effective when compared to the other two resistance genes (WALKER et al., 2004; ZHU et al., 2008). Therefore, the addition of another QTL, in order to increase insect resistance, represents an interesting strategy for biological control in soybean cultivars (ZHU et al., 2008).

Recently, transgenic lines of soybean expressing the *B. thuringiensis* toxin Cry1Ac were tested in the field for their potential resistance against lepidopteran pests. It was demonstrated that Bt toxins could also be used in soybean as a resistance methodology aimed towards *A. gemmatalis*, *P. includens*, and *Hypena scabra* (MCPHERSON e MACRAE, 2009).

3.2 Digestive enzyme inhibitors

The occurrence of proteinase inhibitors (PIs) as defence-related proteins and their role on plant protection are well described in the literature. Since 1947, it has been observed that PIs from soybean were able to inhibit the growth of insect-pests larvae, including the coleoptera *Tribolium confusum* (HAQ et al., 2004; LYSON, 2002; MICKEL e STANDISH, 1947). Later, *in vitro* and *in vivo* bioassays demonstrated that protease inhibitors were also active against other insect species, such as *Anagasta kuehniella*, *Hypera postica* and *Anthonomus grandis* (FRANCO et al., 2003; MACEDO et al., 2003; WILHITE et al., 2000). Hence, as there is no evidence that proteinase inhibitors have toxic or deleterious effects on mammals, they constitute a significant alternative for the development of transgenic crops resistant to insect-pests and nematodes.

Soybean cultivars are predated by several insect species, requiring the application of different insecticide compounds in agriculture. Proteinase inhibitors, such as AKTI (*Albizzia*

kalkira proteinase inhibitor), could, therefore, be a promising choice, as it has been shown to reduce the activity of the beet armyworm *S. exigua* by 57% (ZHOU et al., 2008).

Approaches for the development of genetically engineered soybean lines, which express insecticidal molecules, are also being widely studied. Transgenic tobacco expressing a cowpea trypsin inhibitor (CpTI) showed activity against *Heliothis virescens* larvae (GATEHOUSE et al., 1993), as well as species from *Diabrotica* spp and *Spodoptera* spp (HILDER et al., 1987). However, it was shown that in *Nicotiana tabacum* transgenic plants expressing the barley trypsin inhibitor BTI-CMe, although larvae of *Spodoptera exigua* were susceptible to the proteinase inhibitor, they were able to produce alternative proteins that could maintain survivance of the insect-pest (LARA et al., 2000). This report demonstrated the adaptation development of *S. exigua* over genetically modified tobacco cultures.

The inhibition of nematode digestive enzymes is one type of anti-feeding strategy, exemplified by plant transformation with genes encoding proteinase inhibitors (ATKINSON et al., 2001; LILLEY et al., 1999a; LILLEY et al., 1999b). To this end, there are reports that describe the characterisation of proteinase activities in crude protein extracts of plant-parasitic nematodes (LILLEY et al., 1996; MICHAUD et al., 1996) or the isolation of their proteinase genes (FRAGOSO et al., 2005; FRAGOSO et al., 2009; LILLEY et al., 1997; NEVEU et al., 2003; URWIN et al., 1997a). For example, the production of a genetically modified *Arabidopsis thaliana* with a gene encoding the modified proteinase inhibitor from rice, cystatin Oc-1 delta D86, was able to inhibit growth of *Heterodera schachtii* and *Meloidogyne incognita* females, blocking egg production and pest proliferation. Other reports showed the potential application of anti-feeding strategies based on plant transformation to express proteinase inhibitors (ATKINSON et al., 2001; URWIN et al., 1997b; URWIN et al., 1998), proteinase gene silencing by RNAi (URWIN et al., 2002), *in vitro* inhibition of cysteine proteinase activity using the cognate pro-region of nematode cysteine proteinase (SILVA et al., 2004) and the transformation of soybean roots to express the propeptide (MARRA et al., 2009).

3.3 Defensins: small tools against insect resistance

Defensins are antimicrobial peptides varying from 45-54 amino acid residues stabilised by 3-4 disulfide bridges, which are isolated from different sources, such as plants, mammals, insects and crustaceous (THOMMA et al., 2002). They have been described as important tools for the control of pathogenic fungi, especially *Rhizoctonia solani* and *Fusarium* species, which are important organisms that causes damage in soybean cultivars (OLLI e KIRTI, 2006; WANG e NG, 2007). Therefore, the study of defensins may lead to the development of future transgenic plants encoding peptides toxic to these phytopathogenic fungi.

It was previously demonstrated that a recombinant defensin from mungbean (rVrD1) was able to inhibit the growth of *R. solani* and *F. oxysporum* (CHEN, 2004). The same fungi also had their activity inhibited by another defensin-like peptide, called coccinin, isolated from the seeds of large scarlet runner beans (*Phaseolus coccineus*) (Ngai *et al.*, 2004). Furthermore, Tfgd1, a recombinant defensin from the legume *Trigonella foenum-graecum*, demonstrated similar features, inhibiting the growth of *R. solani* at a low concentration (OLLI e KIRTI, 2006). In addition, *F. oxysporum* growth was strongly affected by the recombinant *Vitis vinifera* antimicrobial peptide (Vv-AMP1), as well as by the peptide from the flowers of *Nicotiana alata,* NaD1 (BEER e VIVIER, 2008; WEERDEN e ANDERSON, 2008). Psd1, a defensin isolated from pea seeds, was also able to decrease growth of the phytopathogen *F. solani* by interacting with the fungus membrane, leading to cell death (MEDEIROS et al., 2010). Many other defensins have been characterised as having antifungal activities, and these can be observed in Table 2.

Studies on the expression of defensins in transgenic model plants are confirming the activity of foreign peptides as an important tool against soybean pathogenic fungi. Hence, using *Arabidopsis thaliana* as an expression system, defensins PDF1.1 and PDF1.3 were produced, purified and analysed against several fungi species, showing activity against two soybean pathogens, *F. graminearum* and *F. oxysporum* (SELS et al., 2007).

3.4 Using spider and scorpion venom toxins as novel insecticidal mediators

Scorpion and spider venoms are being studied for their several physiological and pharmacological effects against pests and pathogens (ROCHAT et al., 1979; ZLOTKIN et al., 1991).

Scorpion toxins seem to have specificity to some insects that cause economic crop losses around the world, including soybean loss. Therefore, such toxins are being evaluated for use in future development of recombinant biopesticides as an alternative strategy to control insect-pests. Several insect-related toxins have been identified from scorpions, isolated from diverse geographical locations (BECERRIL et al., 1995; CREST et al., 1992; NAKAGAWA et al., 1997; ZLOTKIN et al., 1991). In this manner, toxins purified from the venom of the spider *Plectrurys tristis* demonstrated insecticidal activity against *H. virescens*, an insect-pest of soybean cultivars (QUISTAD and SKINNER, 1994). Moreover, a toxin identified from the venom of *Mesobuthus tumulus*, called ButaIT, showed high antagonistic activity towards the insect *H. virenscens* (WUDAYAGIRI et al., 2001).

The interest in developing insecticidal compounds for field application in order to reduce the use of chemical agrotoxics and decrease insect resistance has led to new biotechnology-based approaches. Although not yet used in soybean plants, the insertion of a gene encoding toxin into the genome of a plant or baculovirus is a potential alternative (KHAN et al., 2006; LIMA et al., 2007; STEWART et al., 1991). The fusion of a toxin to the N-terminal of insect lectins has also given successful results. Lectins can act as carrier proteins and direct the fused toxins to the insect haemolymph, causing death (PHAM TRUNG et al., 2006).

3.5 Using gene silencing for nematode control

One of the techniques utilised in soybean biotechnological products is the genetic transformation of cultivars expressing double strand RNAs (dsRNA) in order to drive gene silencing in nematodes. The mechanism of pos-transcription gene silencing using dsRNA is known as RNA-mediated interfering (RNAi), or gene silencing (BOSHER e LABOUESSE, 2000; HUNTER, 2000; KUWABARA e COULSON, 2000; SHARP, 1999). Gene silencing can be either partial – called knckdown – or total – denominated knockout.

Briefly, the RNAi mechanism uses DICER complexes (dsRNA-specific RNase III-type endonuclease), which recognise and digest the dsRNA into siRNAs (small interfering RNAs) that ranges from 21-26 base pairs. These siRNAs flow through four possible pathways. First, siRNAs bind to the RISC complex (RNA-induced silencing complex) to search for and destroy complementary mRNA. Second, siRNAs bind to the RdRp complex (RNA-dependent RNA polymerase), priming mRNAa to synthesise new dsRNAs, and potentialising the entire RNAi process. Third, siRNAs bind to the membrane protein complex SID (systemic RNAi), which spreads out siRNAs to neighbourhood cells and, probably, to all cells, generating a systemic response of gene silencing. Fourth, siRNAs bind to the RITS complex (RNA-induced transcriptional silencing), which drives heterochromatin condensation of homologous regions, directing gene promoter turn-off and, consequently, pre-transcriptional gene silencing.

Researchers have applied RNAi technique in order to evaluate the effect of RNAi on nematode development. This method was already widely used in the free-living nematode *Caenorhabditis elegans*, and its effect is normally gene-specific, systemic, lasting, or even hereditary (ALDER et al., 2003; BARGMANN, 2001; BARSTEAD, 2001).

Several reports use the methodology of *in vitro* dsRNA production and administration of nematodes by incubation (soaking), followed by plant infection for the determination of parasitism, such as the levels of reproduction and survival. Some silenced target genes showed high potential for use in parasitism control, such as: (i) proteinase genes in cyst nematodes *Heterodera glycines* and *Globodera pallida* (URWIN et al., 2002), and root-knot nematode *M. incognita* (SHINGLES et al., 2007); (ii) chitin synthase in *M. artiellia* (FANELLI et al., 2005); (iii) aminopeptidase in *H. glycines* (LILLEY et al., 2005); (iv) cellulases in *G. rostochiensis* (CHEN et al., 2005) and *H. glycines* (BAKHETIA et al., 2007); (v) amphideal secretion protein in *G. rostochiensis* (CHEN et al., 2005); (vi) FMRF-like peptides in *G. pallida* (KIMBER et al., 2007); (vii) pectate lyase, corismato mutase and secretion peptide SYV46 in *H. glycines* (BAKHETIA et al., 2007); and (viii) double oxidase in *M. incognita* (BAKHETIA et al., 2005).

Another strategy commonly applied is the use of transformed plants that express nematode target-gene dsRNAs and their evaluation in the presence of nematode infection. In this way, some plants showed partial-to-complete nematode resistance, as observed for the peptide 16D10 dsRNA expressed in *Arabisopsis thaliana* increasing resistance against *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* (HUANG et al., 2006). Moreover, the major sperm protein dsRNA showed increase of soybean resistance towards *H. glycines* in soybean (STEEVES et al., 2006). Reports also describe the successful use of this technique, such as in the silencing of *M. incognita* genes that encode a splicing factor and an integrase. After nematodes were fed with RNAi transformed tobacco roots, they showed smaller and fewer healthy females when compared to those that were fed the control plants (YADAV et al., 2006). Later, it was demonstrated that the exposure of nematodes to RNAi fragments increased their susceptibility to RNAi delivery, confirming the importance of gene silencing techniques as biotechnological tools to improve plant resistance to pathogenic nematodes (LILLEY et al., 2007). Earlier studies also reported that the silencing of a gene encoding cathepsin L-cysteine (mi-cpl-1) from *M. incognita* was able to decrease the number of fertile females. In this case, the production of eggs was reduced to 40% using only a small fragment of the respective gene (800 bp) (SHINGLES et al., 2007). Moreover, studies of the transcription factor of *M. javanica* in tobacco plants produced satisfactory results, showing that the RNAi technique is an excellent strategy to study plant resistance to phytopathogenic nematodes (FAIRBAIRN et al., 2007).

Recently, it was reported that the use of four different RNAi gene-silencing constructs was able to decrease cyst nematodes in transformed soybean roots (KLINK et al., 2005; KLINK et al., 2009). Furthermore, another study showed that silencing of a tyrosine phosphatise gene and a mitochondrial stress-70 protein precursor from *M. incognita* provided the reduction of gall formation in transformed soybean. It also revealed that nematode development was affected by RNAi constructs (IBRAHIM et al., 2010).

Target-specific RNAi of the *H. schachti* gene *Hg4F01*, a related species of *H. glycines*, expressing an annexin-like protein was also studied in order to identify its function on nematode resistance. *H. schachti* was used in this work because of its effect on *Arabidopsis thaliana*, which could not be performed with *H. glycines*. Hence, using plant-host derived RNAi and the *Arabidopsis*–cyst nematode system, it was demonstrated that the annexin-like protein had a significant effect on plant-nematode interactions. In addition, it was concluded

that the expressed protein, which is also produced in *Arabidopsis*, may mimic plant annexin function (PATEL et al., 2010).

4. Directing transgene expression to attacked tissues

4.1 Promoter isolation and characterisation

Once the Cauliflower Mosaic Virus (CaMV) 35S promoter was isolated and characterised (ODELL et al., 1985), it became the major general-purpose and most widely-used promoter in GM plants (HERNANDEZ-GARCIA et al., 2009). CaMV 35S is the promoter of choice in more than 80% of GM plants (HULL et al., 2002), because it drives constitutive high levels of transgene expression (VENTER, 2007).

Despite the great success of the 35S promoter, there is a scientific interest in discovering new promoters with differentiated and specialised functions. Moreover, the stability and expression pattern of foreign genes driven by the 35S promoter has been tested and questioned (BAKHSH et al., 2009; WESSEL et al., 2001). Therefore, the discovery of plant promoters was essential to drive predictable temporal and tissue-specific expression patterns and high levels of protein production (LU et al., 2008). When evaluating transgenic strategies for nematode control, although widely used, the 35S promoter has certain limitations, such as its poor performance in monocots, and its suppression when feeding nematodes (URWIN et al., 1997c).

Plant promoters used in biotechnology are divided into three categories based on gene expression pattern: constitutive (almost everywhere, every time); spatiotemporal (tissue-specific and/or stage-specific); and inducible (regulated by some specific signal) (POTENZA et al., 2004). Inducible promoters can fit into three categories: endogenous-signal responsive (plant hormones); external, physical-stimuli responsive (abiotic and biotic stresses); and external, chemical-stimuli responsive (PEREMARTI et al., 2010).

Plants have evolved defence mechanisms to combat pests and pathogens. Some defence mechanisms are innate, others are only active when and where there is an attack. These mechanisms, therefore, are dependent on biotic-interaction detection, functionality of signalling pathways, defence-gene expression, cellular- and tissue-defence activation. The expression of defence genes is driven by chemical-inducible promoters, when elicitors produced by pests and pathogens are recognised by plants (PEREMARTI et al., 2010). Similarly, pest wounding generates physical signals which direct expression of defence genes. Both elicitor-inducible and wound-inducible promoters are relatively well-conserved across taxonomic groups, implying wide plant species spectra in transgenic strategies (PEREMARTI et al., 2010).

Plant cellular response to biotic stress - initiated by biochemical signalling cascades that detect pest injury or pathogen invasion - can be carried out by activation and/or inhibition of transcription factors (trans-elements). In turn, they bind to specific DNA sequences (cis-elements) to regulate gene expression. In this way, resistance mechanisms are activated only, or mainly, when and where there is an attack, and achieve a lethal dose. Besides identifying genes to control pests and diseases, the use of such biotic-stress induced promoters could ensure transgene expression primarily in affected tissues, specific to a specific targeted pest or pathogen.

Root-knot and cyst nematodes induce plant cell differentiation to generate nematode feeding sites, giant cells and syncytia, respectively, which act as nutrient sinks. Such cellular modification, coordinated by a complex gene-regulation network, is under examination in

several studies in order to find key transcription factors. Some nematode-induced promoters could represent excellent vectors for the administration of lethal doses to nematode feeding sites in infected roots.

Hence, a soybean heat shock protein 90-like promoter (GmHSP90L) and a soybean polyubiquitin promoter (Gmubi) have shown higher expression levels of the gfp gene than the cauliflower mosaic virus 35S promoter (CaMV35S), when transformed in cotyledons of germinating lima bean (*Phaseolus lunatus*) seeds (CHIERA, 2007). An intronic region (Gumpri) from the Gmubi promoter seems to be essential for increasing gene expression levels. It was demonstrated that the activity of Gmubi promoter was two times higher than CaMV35S promoter when the intron was removed, compared to a five times higher when the intronic region was present. Similar results were also obtained for ubiquitin promoters from other sources. The ubiquitin promoter from rice showed no activity when the intronic region was removed (WANG e OARD, 2003). Also, The expression of the same promoter in tobacco was not only reduced when the intron was not present, but the tissue-specific expression was altered (PLESSE et al., 2001). Moreover, the GmHSP90L promoter showed stronger expression levels than CaMV35S, but the expression level decreased after promoter truncation, revealing that this region might be related to GmHSP90L promoter regulatory elements (CHIERA, 2007). Detailed studies of gene expression at nematode feeding sites have been performed by several research groups, and some nematode-induced promoters have been identified and characterised. The TobRB7 gene (enconding a water channel protein - OPPERMAN et al., 1994), the Hahsp17.7G4 gene - which encodes a heat shock protein (ESCOBAR et al., 2003) - and the E2 gene (enconding the ubiquitin conjugation factor 2 - BIRD, 1996), are al highly expressed in the giant cells of *M. incognita*.

Ubiquitination plays important roles, such as on mediating lipidation, protein activity regulation, protein–protein interaction control, subcellular localisation (MUKHOPADHYAY e RIEZMAN, 2007), transcription control through histone ubiquitination, translation control, DNA repair, regulation of endocytosis and protein trafficking (MURATANI e TANSEY, 2003). Indeed, ubiquitination regulates various aspects of plant life, including disease resistance (ZENG et al., 2006), hormone signalling (ITOH et al., 2003), many developmental processes and cell cycle control (MOON et al., 2004). Thus, the E2 enzyme, a member of the Leubc4 family, has a major role in cellular metabolism and giant cell formation. Because of this, its promoter region was suggested for promoter characterization and use as biotechnological tool for root-nematode control (BIRD, 1996). Therefore, the soybean E2 gene promoter, named UceS8.3, was isolated and characterised for further use in soybean genetic transformation (GROSSI-DE-SA et al., 2008). In the same way, the soybean polyubiquitin gene promoter was isolated and characterised (HERNANDEZ-GARCIA et al., 2009).

Functional genomic studies have been applied to study plant-nematode interactions, in order to identify genes that were up-regulated and down-regulated at nematode feeding sites. The use of microarray hybridisation, serial analysis of gene expression (SAGE) and proteomic methodologies (FRANCO et al., 2010; MEHTA et al., 2008) achieved gene expression analyses at genomic scale, while quantitative real time PCR, *in situ* hybridisation and Laser Capture Microdissection techniques provided more accuracy in comparing differences in promoter expression.

The use of novel promoters for gene expression control can be widely used in genetically modified soybean in order to direct the foreign gene to a specific tissue attacked by a certain pest or pathogen. In this way, some promoters identified in soybean have been studied in model plants and compared with other existing promoters from other sources.

Hence Khan and colleagues (2004) made comparisons of 1,300 cDNAs from soybean infested with *H. glycines* at 2 dpi (Table 4). Klink and colleagues (2005) used laser capture microdissection to collect syncytia of soybean under 8-dpi infestation with *H. glycines*. A total of 800 cDNAs were sequenced, assembled as 174 consensus sequences, where 6 were up-regulated, 1 was down-regulated and 5 were both activated or inhibited, as confirmed by qRT-PCR. Later, Aklharouf and colleagues (2006), using microarray chips with more than 6,000 cDNA spots, detected hundreds of differentially expressed genes at from 6 hours (hpi) to 8 days post-infection (dpi). ITHAL and colleagues (2007a) simultaneously compared infested and non-infested soybean roots and different development stages of *H. glycines* at 2, 5 and 10 dpi. The microarray analysis of 35,611 soybean genes and 7,431 nematode genes detected 429 plant genes and 1,850 nematode genes with differential gene expression. ITHAL and colleagues (2007b) made microarray hybridisations to study syncytia development from 0 to 10 dpi. Using chips with 35,611 soybean genes, they observed 1,765 genes with changes in expression pattern until 2 dpi, when 1,116 genes were up-regulated, while 649 genes were down-regulated.

5. Perspectives on GM soybean for insect-pests and diseases control

The glyphosate-tolerant GM soybean alone corresponds to 52% of all biotech crops planted world area. Indeed, considering soybean, herbicide tolerance has still been the major aimed trait, with around ten novel varieties showing tolerance to different chemical compounds in their final steps of R & D pipeline to commercial events.

However, there is an obvious need and seed market demand for insect-pests and plant-pathogens resistance traits. In a very near future, the first GM soybean resistant to insect-pests and nematode will be available as single traits or together with herbicide tolerance (stacked traits).

In that way, several biotechnological strategies and their candidate genes have been tested in order to induce resistance to various pests and diseases, including the tissue over-expression of genes driven by specific plant promoters. Hence, it is expected to observe, in a near future, the production of soybean and its processed products with less or none agrotoxic residues or micotoxins from opportunistic fungi, as it has been already seen by the GM maize containing a Bt toxin. Consequently, it is also expected that further GM soybean traits enable a relevant decrease of costs with chemical pesticides, the enhancement of soybean quality and crop production, as well as the maintenance of a non-poluted and biodiverse environment.

Recenlty, the Academy of Science from the Vatican announced their support on the production of transgenic plants. The report was published in an International Scientific Journal and signed by 40 specialists – including 7 from the Vatican itself. Among other things, the report described the need for development of new agricultural technologies in order to decrease malnutrition and starvation that surrounds 1 billion people of the world (Potrykus *et al.*, 2010). This announcement brought new perspectives for biotechnology, once that now religion and science are walking together for the improvement of food supply and the development of new techniques into plant transformation. Therefore, it is expected that, in future, more advanced strategies provide the cultivation of several transgenic crops with diverse resistances towards pests, pathogens and environment conditions, with the approval for consumption not only from Inspection Organizations, but from the entire society.

Biologic process or predict gene function (Protein)	Time pos infestation (analysis technique)	Reference
▲ Defence ▲ Stress response (peroxidase; SAM22) ▲ Carbohydrate metabolism ▲ Cellular signalling ▲ Unknown	2 dpi (Microarray)	(KHAN et al., 2004)
▲ Defence (SRG2) ▲ Water channel (GmPIP2,2) ▲ Cell skeletal (GmTubA1; GmTubB4) ▲ DNA binding (MYB-related protein) ▼ Protein catabolism (ubiquitin)	8 dpi (EST library)	(KLINK et al., 2005)
▲ Defence ▲ Wounding response ▲ Transcription factor (WRKY) ▲ DNA duplication ▲ RNA transcription ▲ Protein translation ▲ Unknown	6 hpi, 12 hpi 1, 2, 4, 6 and 8 dpi (Microarray)	(ALKHAROUF et al., 2006)
▲ Defence ▲ Stress response (peroxidase) ▲ Primary and secondary metabolism ▲▼ Cell wall modification ▲ Plant development ▲ Cellular signalling ▲ Transcription factor (WRKY; bZIP; ERF; MYB)	2, 5 and 10 dpi (Microarray)	(ITHAL et al., 2007a)
▲ Disease related ▲ Primary and secondary metabolism ▲▼ Cell wall modification ▲ Lignin and suberin biosynthesis ▲▼ Transporter of sugar, metal ion and amino acid ▲▼ Auxin related ▲▼ Ethylene related ▼ Cytokinin related ▼ Gibberellin related ▼ Jasmonic acid biosynthesis ▲ Development related (PHAP2A) ▼ Water channel (Rb7)	2, 5 and 10 dpi) (Microarray)	(ITHAL et al., 2007b)

Differential expression of individual genes, relative to non-infested plants, at different times was associated to predicted gene function and biologic process. The arrows represent predicted biological process activation (▲), inhibition (▼) or undetermined (▲▼), considering the results of individual up- or down-regulated genes.

Table 4. Biologic process modification during infestation of *G. max* plants by *H. glycines*.

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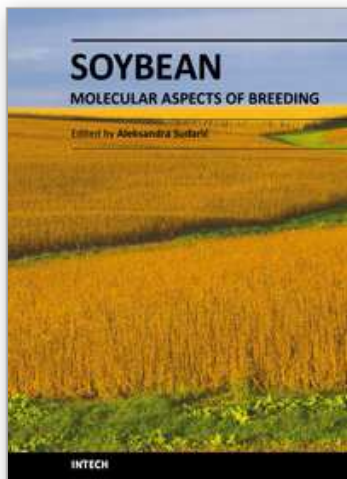
Soybean - Molecular Aspects of Breeding

Edited by Dr. Aleksandra Sudaric

The book Soybean: Molecular Aspects of Breeding focuses on recent progress in our understanding of the genetics and molecular biology of soybean and provides a broad review of the subject, from genome diversity to transformation and integration of desired genes using current technologies. This book is divided into four parts (Molecular Biology and Biotechnology, Breeding for Abiotic Stress, Breeding for Biotic Stress, Recent Technology) and contains 22 chapters.

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