CURRENT REVIEW

Plant Immunity Inducer Development and Application

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Plant immunity inducers represent a new and rapidly developing field in plant-protection research. In this paper, we discuss recent research on plant immunity inducers and their development and applications in China. Plant immunity inducers include plant immunity-inducing proteins, chitosan oligosaccharides, and microbial inducers. These compounds and microorganisms can trigger defense responses and confer disease resistance in plants. We also describe the mechanisms of plant immunity inducers and how they promote plant health. Furthermore, we summarize the current situation in plant immunity inducer development in China and the global marketplace. Finally, we also deeply analyze the development trends and application prospects of plant immunity inducers in environmental protection and food safety.

Recently, environmental pollution and food safety issues resulting from the overuse or inappropriate use of chemical pesticides have attracted much attention worldwide (Dai 2013). To reduce the use of chemical pesticides, governments are searching for nontoxic and technological alternatives. Strategies that enhance plant immunity and promote healthy plant growth have great potential for avoiding plant diseases, thereby reducing the need for pesticides to prevent infestations and diseases (Jones et al. 2013). Therefore, techniques to boost plant immunity represent a new and rapidly developing field of research and development in China.

Immunity inducers are a class of immune-active compounds that can induce systemic acquired resistance in plants. They can be divided into nonbiologically and biologically active molecules, depending on their source. Nonbiologically active molecules mainly include synthetic plant defense elicitors, such as jasmonic acid analogs and 2,6-dichloro-isonicotinic acid (Bektas and Eulgem 2015). Biologically active molecules are active small molecules produced during the interaction between a pathogen and its host and include metabolites, oligosaccharides, glycoproteins, glycopeptides, proteins, polypeptides, lipids, and other cellular components (Table 1). These inducers are recognized by receptors on the surfaces of plant cells and trigger plant defense responses, resulting in systemic resistance (Heese et al. 2007). Here, we discuss plant immunity inducers from biological sources, mainly including plant immunity-inducing proteins, oligosaccharides, and microbial inducers. Microbial inducers refer to types of microorganisms that can induce plant immunity responses, including fungi and bacteria (e.g., members of genera *Trichoderma* and *Bacillus*) (Sharma;

2014). At present, research in the world of plant immunity inducers is mainly focused on hypersensitive proteins, seaweed, and *Trichoderma harzianum*. The main products in use include harpin proteins, seaweed liquid fertilizers, seaweed powder, and biocontrol agents. The development and application of plant immunity inducers is important to maintain healthy crop growth, reduce the occurrence of pests and diseases, and reduce the use of chemical pesticides. Therefore, plant immunity inducers represent a novel strategy to reduce pesticide residues in agricultural products, improving quality and safety, and are experiencing rapid development. In this review, we discuss the development and application of plant immunity inducers.

DEVELOPMENT OF PLANT IMMUNITY INDUCERS

Plant immunity-inducing proteins.

Scientists first observed the phenomenon of plant resistance in 1963, but not until 1992 was it confirmed plant resistance was induced by protein material in the fire blight bacterium, named the hypersensitive protein (harpin) (Wei et al. 1992). Harpins can increase crop yield, improve crop quality, and enhance resistance to viral diseases and aphid infestation (Baker et al. 1993). Messenger (harpin protein), which is produced by plant-pathogenic bacteria, stimulated the development of agricultural biotechnology products and a 3% particle bioagent using this protein was invented by W. Zhongmin at Cornell University in 1992 (Grisham 2000). In 2000, Messenger, which had passed the pesticide residues test of the United States Environmental Protection Agency (EPA), was registered in the United States by Eden Biotechnology Company and was permitted for use on all crops. In 2001, this product won the Presidential Green Chemistry Challenge Award issued by the EPA and was described as the first green revolution in plant protection and agricultural products safety. It has since been used on tobacco, vegetables, and fruits in the United States, Mexico, Spain, and other countries. Additionally, the Institute for the Control of Agrochemicals, Ministry of Agriculture of China approved a provisional registration certificate in 2001. In 2007, this product was permitted to be used on tomato, pepper, tobacco, and rape. Because of its unique mechanism and clear effect on disease and insect resistance, Messenger has received extensive attention from experts and scholars and agricultural dealers.

Plant immunity-inducing proteins are being rapidly developed in China. The protein-gene-protein pattern, which refers to a series of techniques for screening new proteins from microbial sources, including extraction and purification, gene cloning and expression, and protein structure and function analysis, was proposed for finding new plant immunity-inducing proteins (Wang et al. 2011). Based on this method, ten proteins and related gene-cloning procedures are now protected by Chinese

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patents. Compared with the traditional strategy, in which potential plant immunity-inducing proteins are identified by screening genetic libraries, the protein-gene-protein pattern avoids repeated screening and splicing from gene libraries and repeated verification and makes it easier to obtain a complete protein with biological activity. This technical strategy has accelerated the process of protein drug discovery and development. Innovative screening procedures have resulted in the isolation of various immunity-inducing proteins (PeaT1, Hrip1, MoHrip1, MoHrip2, PemG1, PevD1, BcGs1, PebC1, PeBL1, and PeBA1) from a wide range of pathogens, including *Alternaria tenuissima*, *Magnaporthe oryzae*, *Verticillium dahliae*, *Botrytis cinerea*, *Brevibacillus laterosporus*, and *Bacillus amyloliquefaciens* (Chen et al. 2012, 2014a and b; Kulye et al. 2012; Peng et al. 2011; Wang et al. 2011, 2015, 2016; Zhang et al. 2011b, 2014, 2015). These proteins have the potential to be developed into new protein pesticides.

Several studies have focused on establishing a multifunction, multi-index evaluation system for plant immunity-inducing proteins. Such a system can help to elucidate the mechanism of protein-induced plant resistance and enhanced plant growth as well as uncover the molecular basis for these mechanisms and related signal transduction pathways (Wang et al. 2010). To reveal the mechanisms of plant immunity-inducing proteins, researchers have analyzed the binding sites of plant immunity-inducing proteins in the tobacco cell membrane, the activation of early plant defense signals (pH increases and production of H₂O₂ and nitric oxide [NO]), and the expression levels of defense-related genes, proteins, and protein kinases (Chen et al. 2012, 2014a and b; Wang et al. 2011; Zhang et al. 2014). Based on these results, a multifunction, multi-index evaluation system has been established, which includes measurement of the hypersensitive response, *Tobacco mosaic virus* (TMV) resistance, oxygen bursts, the expression of defense-related genes, NO production, changes in extracellular fluid pH, and changes in plant growth (Kulye et al. 2012; Liu et al. 2016; Wang et al. 2011; Zhang et al. 2015).

In China, new technologies have been developed to produce plant immunity-inducing proteins with high efficiency at low cost. For example, the culture conditions (e.g., medium, temperature, pH, dissolved oxygen) for natural strains of *Alternaria*

alternata were optimized and, then, these parameters were adjusted in the scale-up phase. The optimization resulted in an increase in protein yield from 5.17 to 133.7 g per liter (Jin et al. 2009). Other studies have focused on postfermentation processing, including protein extraction and preparation processes. The development of a colloid mill extraction process has greatly increased protein extraction efficiency, as compared with that achieved using ultrasonic, high pressure cell-rupture or centrifugal separation methods (Vishwanathan et al. 2011). Fermentation liquid treatments have been developed to retain peptide and oligosaccharide metabolites that function synergistically as plant immunity inducers and to protect their activity during processing (Cino 1999; Carresi et al. 2006). Such research has resulted in the development of large-scale, highly efficient, low-cost production systems for plant immunity-inducing proteins (Buensanteai et al. 2010).

New types of chitosan oligosaccharides.

The use of seaweed and its extracts in the growing and breeding industry has been recognized by many international organizations and governments (Leonard et al. 2012). The European Union IMO certification, OMIR (Organic Materials Review Institute) certification, and Chinese organic food technical specifications clearly allow seaweed products as soil fertility and improvement materials, for pest control, and in crop livestock feed additives (O'Sullivan 1947). In recent years, more and more attention has been paid to the application of seaweed and its extracts in agriculture, and processing technologies and application levels have continuously improved (Arioli et al. 2015; Senthilkumar et al. 2007). Since the 1990s, research on seaweed and its extracts for fertilizer applications has progressed rapidly. A large number of scientific research institutes, universities, and enterprises have actively explored extraction processing methods, application effects, and mechanism of action in algae. According to statistics, more than 40 enterprises involved in domestic seaweed fertilizer processing are currently registered with the Ministry of Agriculture. In 1949, seaweed liquid fertilizer was first produced in the United Kingdom. There are two kinds of seaweed liquid fertilizer, one is extracted from seaweed ash and the other from fresh seaweed, which is the type used by most countries. The development of seaweed extracts has experienced three stages: decaying algae, seaweed, and gray (powder)-seaweed extract (Krajnc et al. 2012; Zodape et al. 2008). Furthermore, besides seaweed liquid fertilizers, many other products are available, such as fertilizer with seaweed powder and seaweed essence.

Oligosaccharides have been used as plant immunity inducers in China for many years, and there have been a large number of studies on their uses and functions. Natural chitosan oligosaccharides and trehalose are effective plant immunity inducers and have been shown to enhance disease resistance in a variety of crops (Tsutsui et al. 2015). The plant-pathogen interaction process results in the release of cell-wall oligosaccharides that mediate plant immune activation to increase disease resistance, and this effect can be exploited and developed for use in new agricultural systems (Miya et al. 2007; Yin et al. 2016). Research and development on oligosaccharide agents has become a hot topic in the field of plant protection worldwide, and the number of commercially available products based on such compounds is increasing.

Chitosan is a component of the cell walls of some pathogenic bacteria, and degradation products of this polymer that are released during plant-pathogen interactions can trigger plant defense responses, leading to increased disease resistance. Chitosan has been used both as a biostimulant to promote plant growth and abiotic stress tolerance and to induce pathogen resistance (Pichyangkura and Chadchawan 2015). The plant defense system induced by chitosan is triggered via the NO signaling

Table 1. Plant immunity inducers that have obtained a pesticide registration certificate

No.	Species	Source	Function
1	Messenger	America	Fungicide
2	Benzothiadiazole	Swiss	Fungicide
3	KeyPlex humic acid	America	Plant growth
4	Probenazole	Japan	Fungicide
5	Serenade <i>Bacillus subtilis</i>	America	Fungicide
6	Laminarin	France	Fungicide
7	Oxycom	America	Fungicide
8	Chitosan	Korea	Plant growth regulator, preservative, fungicide
9	Actigard	Swiss	Fungicide
10	NCI	Japan	Fungicide
11	Pyraclostrobin	Germany	Plant growth regulator, fungicide
12	Plant Activator Protein	China	Plant growth regulator
13	trans-Abscisic Acid	China	Plant growth regulator
14	ATaiLing	China	Antiviral agent
15	Oligosaccharins	China	Inducer, Fungicide
16	Methiadinil	China	Plant activator; antiviral agent
17	Lentinan	China	Plant growth regulator, antiviral agent
18	Validamycin	China	Fungicide
19	Matrine	China	Fungicide

pathway (Raho et al. 2011; Zhang et al. 2011a). Several oligosaccharides have been successfully produced on a commercial scale. At present, 11 such compounds are registered as bio-pesticides in China, and their combined application area in China is at least 1.3 million square miles. These compounds can increase agricultural production by improving the yield and quality of crops. Chitosan is found in many inexpensive raw materials and is easily degraded, many products have been developed using chitosan and its derivatives (Ravi Kumar 2000; Yin et al. 2016). In China, researchers have mainly focused on developing biological preparations of chitosan and proteins to add to mixed pesticide formulations. Chitosan has also been certified by the South Korean Ministry of Agriculture and Forestry as an environmentally friendly crop protection agent. In China, some sugars have been registered as plant immunity inducers, and more than 80 products have been developed for use alone or as components of chitosan oligosaccharide products.

Besides chitin and chitosan, many other polysaccharide components of plant and pathogen cell walls are released during plant-pathogen interactions, including pectin in plant cell walls and glucan in fungal cell walls (van Loon et al. 2006). In China and many other countries, researchers have explored bioactive pectin and its degradation products (e.g., oligo polygalacturonic acid) and fungal cell-wall polysaccharides and their degradation products (Combo et al. 2012). These products have been shown to strongly induce disease resistance in some plants. The mechanism of oligomeric galacturonic acid is relatively clear; however, because of the complex structure of the cell wall, the lack of clean production technologies, and the high cost of development, galacturonic acid has not yet been formulated into commercially available products. To further validate the use of oligosaccharides as plant immunity inducers, more in-depth studies on their mechanisms of action were conducted and the concept of a “sugar chain plant vaccine” was proposed. This refers to the application of chitosan oligosaccharides to a wide range of crops to improve their resistance (Wang et al. 2014). Taken together, previous studies that have screened and optimized glycosidase products, investigated the controllable preparation of oligosaccharides, and established a multifunctional and multi-index evaluation system have laid a foundation for the development and application of these products.

Microbial inducers.

At present, more than 60 countries in the world use more than 100 kinds of biological products containing wood mold ingredients (Xu 2008). Fungal fungicides, the main benefit of which is soil microbial control of root diseases and biological control of soil-borne diseases, produce no pollution or resistance and can be used in organic products. These fungi can form a protective layer on the roots and promote plant growth (Soliman et al. 2013). *T. harzianum*, for example, provides life-long benefits for a one-time investment. Compared with other *Trichoderma* products, it has many advantages, including a wide antimicrobial spectrum and wide applicability to crops, providing resistance to high temperature, high humidity, and high salt, other growth-promoting effects, and pesticide compatibility, and is especially suitable for crop seedbed or seedling root irrigation of vegetables, fruits, ornamental plants, landscape tree seedlings, and economic crops (Perazzolli et al. 2011). *T. harzianum* T22 has been used to coat maize seeds to prevent disease caused by *Colletotrichum graminicola* (Harman et al. 2004).

Microorganisms can induce plant immune responses and trigger plant resistance to subsequent pathogen attacks. The *Trichoderma* fungus has a long history of use as a biocontrol microorganism and there has been much research and development to formulate strains into commercial products (Harman et al. 2004; Perazzolli et al. 2011). Such products are now widely distributed on a global

scale. *Trichoderma* spp. are beneficial microorganisms that are widely distributed in soils. A number of manufacturers in China have registered *Trichoderma* strains and products and there is a growing body of research on its ability to induce resistance in plants. Researchers have confirmed that *Trichoderma* spp. and their metabolites can induce plant immunity and improve crop resistance (Djonović et al. 2007). Previous studies have suggested that *Trichoderma* spp. secrete many proteins, including a serine protease, a 22-kDa xylanase, a chitin deacetylase, chitinases, Chit42, SnodProt1 proteins (Sm1, and Epl1), lipopeptides, patulins, and an avirulence (AVR) protein, which not only enhance plant immunity but also promote plant growth (Djonović et al. 2006; Perazzolli et al. 2011; Seidl et al. 2006). Microorganisms including various bacteria (e.g., *Bacillus subtilis*) and fungi (e.g., *Trichoderma* spp.) can induce a microbe-triggered immunity response in plants. Some specific elicitors, for example, AVR proteins and the products of resistance genes, participate in specific interactions and induce immune responses in plants, namely, the effector-triggered immunity reaction (Zhang and Zhou 2010). Thus, such organisms have broad applications in inducing resistance and promoting plant growth.

Five main strains of *Trichoderma* spp. are used as microbial inducers of plant immunity—*T. virens*, *T. viride*, *T. harzianum*, *T. asperellum*, and *T. aureoviride*. *Trichoderma* produces the elicitor Sm1/Epl1, which is a typical plant immunity-inducing protein belonging to the cerato-platanin family (Djonović et al. 2006; Seidl et al. 2006). Studies have suggested that Sm1, which contains a β -pleated sheet, increases the expression of defense-related genes and proteins, leading to systemic resistance in plants (Djonović et al. 2006, 2007). The plant immunity induced by *Trichoderma* spp. is the same as that induced by Sm1.

Most of the recent research on *Trichoderma* spp. has been conducted in China. Based on a series of methods including mutant construction, analysis of protein activity and target protein functions, and proteome and transcriptome analysis, the mechanism of *Trichoderma* spp. has been elucidated. The results demonstrated that some secreted proteins, such as Sm1, cellulase, and other hydrophobic proteins, could trigger defense responses in plants (Djonović et al. 2006). Furthermore, Liu et al. (2009) found that a spore suspension of *T. harzianum* nf9 and a crude extract of wood xylanase both induced resistance to rice sheath blight and that rice plants treated with nf9 showed increased dry weight and a higher root/shoot ratio.

In previous studies, the small hydrophobic protein hyb2 was obtained from the T4 strain of *T. asperellum* and the full-length cDNA and genomic DNA sequences of *hyb2* were cloned. Structural analysis revealed that hyb2 was a hydrophobic protein that contained two adjacent β -hairpin core beta-barrels and an α -helix structure and belonged to the type II hydrophobic protein family (Gulijimila et al. 2012). Subsequently, the chitinase activity of hyb2 was enhanced by gene recombination technology (Cong et al. 2012). Furthermore, cDNA libraries of *T. asperellum* T4 and *Globosum chaetomium* W7 have been constructed (Wang et al. 2013).

COMMERCIALIZATION OF PLANT IMMUNITY-INDUCING PROTEINS

Previous studies have shown that the fungus *A. alternata* can enhance plant disease resistance. The plant immunity-inducing protein PeaT1, which was obtained from *A. alternata*, improved plant resistance against viruses, reducing viral disease by 70 to 80% compared with controls in a field trial, and increased grain yields by at least 10% (Zhang et al. 2011b). A multi-index and multifunctional evaluation system that included induction of reactive oxygen species, expression of defense-related genes, resistance to TMV, and measurements of chlorophyll content

showed that PeaT1 had a dual function in increasing plant disease resistance and improving plant growth (Zhang et al. 2010, 2011b). PeaT1 was then produced on a large scale, using the newly developed colloid mill extraction process. Based on the above results, ATaiLing, which mainly consists of PeaT1, was created, and the first factory for the three-stage fermentation process was built in Henan Province, with an annual output capacity of 800 tons. The activity of the protein was stable for at least two years, allowing robust trials to confirm its low toxicity and its efficiency as an antiviral protein. Environmental assessments confirmed that the protein was nontoxic and environmentally friendly, showing no food security issues to humans or other animals. Consequently, there has been an upsurge in research on the concept of proteins as plant vaccines. ATaiLing received an award in 2015 as the top-selling crop protection product in China and its application area was more than 10 million acres in its first two years after release. In 2015, sales of ATaiLing reached more than 250 tons, equal to US\$10.16 million, when it was first launched onto the domestic market (Fig. 1). ATaiLing is one example of the commercialization of an effective product to control crop diseases in China, is considered very effective by farmers, and has passed the certification for organic products.

As a leading company in crop protection and life sciences, the American company Arysta LifeScience (ALS) markets crop protection products in more than 125 countries. An agreement between the Institute of Plant Protection (IPP) in China and ALS gave ALS access to protein technologies and all related end-use formulations as well as the right to develop new solo formulations and mixtures. This met the requirement of ALS to increase investment in bio-control products and was conducive to providing sole active ingredients and active ingredient combinations to overseas growers. ALS contacted the IPP in 2014 and initiated a comprehensive evaluation of the global market, named the Green Tea Project, in January 2015. Multibatch trials were carried out at a screening station in Saint Malo, France. Samples were analyzed in the laboratory and tested in field conditions in eight countries in the Americas, Africa, and Asia. The results from 35 tests indicated that plant immunity-inducing proteins effectively promoted plant root growth, enhanced plant immunity, controlled plant diseases, left no residues, and were environmentally friendly.

In February 2016, ALS became the sole overseas agent of ATaiLing after strict analyses and assessments. This is the first case of the sale of a Chinese agricultural crop disease control/biological pesticide to the United States, and the two parties (IPP and ALS) expressed expectations and confidence for cooperation. It is hoped that this cooperation will become a successful model for the commercialization of Chinese research, and that it will begin a new era in international cooperation.

Fig. 1. Development of antiviral agent ATaiLing.

Consequently, ATaiLing has become the first Chinese plant immunity-inducing protein pesticide to be launched into the international market.

APPLICATIONS OF PLANT IMMUNITY INDUCERS

In recent years, there has been increasing interest in the development and application of plant immunity inducers as green biological control agents. Such agents improve plant disease resistance so that crops are less vulnerable to diseases, reduce pest damage, and reduce or avoid the use of chemical pesticides, thereby addressing problems of environmental pollution and product safety (Zhang et al. 2011b). New technologies and methods have been developed to produce these agents cheaply and on a large scale. Plant immunity inducers not only prevent disease but, also, promote healthy plant growth so that crops are more tolerant to insect damage (Harman et al. 2004). Therefore, they represent a comprehensive approach to pest and disease management.

Around the world, more and more attention has been focused on food safety and environmental security, and many countries restrict the use of chemical pesticides. Plant immunity inducers can be used safely on fruit and vegetable crops and trigger the natural plant defense system to prevent or control diseases (Perazzolli et al. 2011). Because plant immune inducers are natural products, they have a high degree of acceptance among farmers and consumers, which is an important factor in establishing and growing this industry.

Plant immunity inducers can stimulate plant systemic acquired resistance (Zhang et al. 2011b). Chemical compounds that have this effect include benzene thiadiazole, tiadinil, 2,6-dichloro-isonicotinic acid, N-methyl cyanide 2-chloroethyl isonicotinamide, allyl isothiazole (probenazole), jasmonic acid methyl ester, and the bacterial iso-thiazide amine isotianil (Bai et al. 2011; Gozzo 2003; Yoshioka et al. 2001). Derivatives containing benzene thiadiazole cations or anions have different physical and antibacterial properties but retain the ability to induce systemic acquired resistance in plants; however, because of the high cost of production, these derivatives have not been released commercially. Tiadinil has been studied for control of rice blast disease and was shown to induce expression of resistance genes in tobacco (Noguchi et al. 2006). Thiazide amines displayed the ability to stimulate rice resistance against rice blast disease (Miedema et al. 1999; Pye et al. 2013). These two compounds have been used to control diseases in Japan.

New multifunctional plant immunity inducers, such as plant immunity-inducing proteins, oligosaccharides, abscisic acid, *Bacillus subtilis*, and *Trichoderma* spp., have been registered in China and are becoming more popular. As the application area has increased, it has become clear that the distinct advantage of bio-inducers over traditional bactericides is that bio-inducers do not directly kill the pathogenic microorganism but promote plant growth and strengthen the plant's own immune system, resulting in broad-spectrum disease resistance and stress resistance. The Chinese Academy of Agricultural Sciences has taken the lead in developing the latest research results into commercial products, in collaboration with industry. A plant immunity-inducing agent (ATaiLing) registered as a bio-pesticide in 2014 has been popular with farmers and field trials have shown that it reduced viral diseases by more than 70% and increased the yields of vegetable, fruit, and tea crops by more than 10%.

FUTURE PROSPECTS OF PLANT IMMUNITY INDUCERS

At present, research on plant immunity inducers is focused on finding new biological pesticides and on working with

industry to develop such compounds and organisms into safe, inexpensive products for commercial release. This represents a new strategic industry with great prospects for development. The International Plant Protection Study has focused on the basic theory of plant protection and has made important contributions to this field. This has greatly enhanced the status and strength of China in the field of plant immunity and contributed to the sustainable development of agriculture worldwide. The use of plant immunity inducers has great significance for crop health, ecological and environmental protection, and food safety.

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