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PRs proteins and their Mechanism in Plants

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ABSTRACT: Fungi are far more complex organisms than viruses or bacteria and can developed numerous diseases in plants that cause loss of big portion of the crop every year. Plants have developed various mechanisms to defend themselves against these fungi which include the production of low molecular weight secondary metabolites, proteins and peptides having antifungal activity. Pathogenesis-related proteins (PRs) (initially named 'b' proteins) have focused an increasing research interest in view of their possible involvement in plant resistance to pathogens. This assumption flowed from initial findings that these proteins are commonly induced in resistant plants, expressing a hypersensitive necrotic response (HR) to pathogens of viral, fungal and bacterial origin. PRs have been defined as 'proteins encoded by the host plant but induced only in pathological or related situations', the latter implying situations of non-pathogenic origin. In this review, brief information like biochemistry, source, regulation of gene expression, mode of action of defense mechanism of various pathogenesis-related proteins is investigated.

Keywords: HR, Pathogenesis-related Proteins, Plant defense, Systemic acquired resistance.

INTRODUCTION

The PR-proteins were defined as 'proteins coded by the host plant but induced only in pathological or related situations' (Antoniw *et al.,* 1980). However, related proteins were identified that accumulate in normal (uninfected) plants in certain tissues or developmental stages. These proteins are referred to as 'PR-like' proteins (Van Loon, 1999). In some situations, a gene for a PR-like protein expressed in a developmentally controlled manner may be inducible in some other plant tissue in response to stresses. Thus, the distinction between a PR-protein and a PR-like protein may be less clear-cut in some situations. The PR-proteins are identified easily in cell extracts of infected plants (Van Loon and Van Kammen, 1968, Gianinazzi and Vallee, 1969). In fact, they are quite prominent in acid extracts of infected plants. Perhaps because of the need to function in a hostile environment, most PR-proteins show pH- and thermal stabilities and are quite resistant to proteolysis. In addition, the accumulation of a subclass of PR-proteins (typically the acidic forms) in the apoplastic fluid (extracellular compartment) has simplified the purification of several PR-proteins. Furthermore, the availability of several affinity chromatography procedures for the isolation of some PR-proteins (chitin and curdlan columns, for example) has been very useful in their rapid purification. Thus, many of the PR-proteins have been purified easily to homogeneity and used for the preparation of antibodies specific for each group. The availability of antibodies also has led to the cloning of the corresponding cDNAs and genes for PR-proteins. Thus databases contain several hundred sequences for PR-proteins from diverse plants. From the major cereals, cDNA or genomic clones for a large number of PR-proteins have been isolated Association of pathogenesis-related proteins with induced resistance

The notion that the enhanced resistance apparent upon challenge inoculation depends on the same defense mechanisms as expressed after primary infection led to the identification of common metabolic alterations induced systemically in response to local infection. Whereas induction of phytoalexins and cell wall rigidification are local reactions, accumulation of pathogenesis-related proteins (PRs) extends into non-inoculated plant parts that, upon challenge, exhibit acquired resistance (Van Loon and Van Kammen, 1970, Ryals *et al.,* 1996). The proteins themselves are not transported from the primary inoculated leaves, as demonstrated elegantly by (Gianinazzi and Ahl, 1983) through the analysis of reciprocal grafts of Nicotiana species expressing electrophoretically different proteins. While a link between PRs and acquired resistance in virus-infected tobacco was immediately hypothesized (Van Loon and Van Kammen, 1970, Kassanis *et al.,*1974, Van Loon, 1975, Fraser, 1982) pointed out that PRs became apparent in non-inoculated leaves distinctly later than acquired resistance appeared manifest. However, in tissues already primed to express PRs, challenge inoculation might lead to their earlier and faster accumulation. Moreover, a hybrid between *N. glutinosa* and *N. debneyi* constitutively expressed PRs and was highly resistant against TNV (Ahl and Gianinazzi, 1982). Induction of PRs has since been found to be invariably linked to necrotizing infections giving rise to SAR, and has been taken as a marker of the induced state (Ward *et al.,*1991, Uknes *et al.,*1992, Kessmann *et al.,*1994).

This notion has been reinforced by the characterization in Arabidopsis of mutants that either are compromised in both the production of PRs and the induction of SAR (npr1; Cao *et al.*,1994, nim1; Delaney *et al.*,1995), or are constitutive expressers of PR genes as well as SAR (cpr1; Bowling *et al.*,1994). PRs have been defined as plant proteins that are induced in pathological or related situations (Van Loon *et al.*,1994). Although they are implicated in plant defense, they have not been identified because of their anti-pathogenic action, but solely because of their accumulation in infected plants. Eleven families of PRs have now been officially recognized (Van Loon *et al.*,1994), but additional pathogen-induced proteins with potential anti-pathogenic action keep being described (Broekaert *et al.*,1995). PRs have been identified in at least nine plant families, with those in tobacco and tomato characterized best. Now, it is known that they comprise four families of chitinases (PR-3, -4, -8 and -11), one of β-1,3-glucanases (PR-2), one of proteinase inhibitors (PR-6), and one specific peroxidase (PR-9), as well as the PR-1 family with unknown biochemical properties, the thaumatin-like PR-5 family, and the birch allergen Betv1-related PR-10 family. Not all families are represented in any plant species, but each family may comprise several members. Together the PRs form a set of pathogen-induced proteins that may be considered as stress proteins. In the past decades it has become evident that plants, when exposed to various environmental stresses, respond by synthesizing sets of specific proteins. Well-known are the heat-shock proteins, that appear to be common to all living organisms, and are transiently induced when ambient temperature exceeds some critical limit (Vierling, 1991). Different sets of proteins are induced by e.g. drought stress or freezing temperatures. For instance, during cold acclimation hardy cultivars of alfalfa synthesize a number of proteins that supposedly function in reducing the deleterious effects of low temperature on plant membranes. The plant hormone abscisic acid (ABA) induces a partly similar set of proteins and increases resistance to freezing stress, indicating that acclimation is hormone-controlled (Mohapatra *et al.*,1988, Heino *et al.*, 1990). Similar proteins are induced by ABA during the acquisition of desiccation tolerance in developing seeds and upon drought stress of leaves (Skriver and Mundy, 1990). PRs may be considered as stress proteins produced in response to, particularly necrotizing, infections by viruses, viroids, fungi and bacteria, and thought to function in the acquired resistance against further infection (Van Loon, 1989). However, in contrast to most other types of stress proteins, they accumulate in plant tissues to levels that are easily detectable on gels by general protein stains. Why these inducible PRs may individually reach up to 1% of the total soluble protein in leaves, is unclear. Some of the PRs possess potential antipathogenic activities (Linthorst, 1991, Van Loon *et al.*, 1994).

Chitinases, together with glucanases, could be directed against fungal cell walls and, perhaps, insects. Insects are likely to be affected by proteinase inhibitors. Bacteria may be inhibited by the PR-8 family of chitinases, which also possesses lysozyme activity. The PR-9 peroxidase is of the lignin-forming type and could be involved in the strenghtening of cell walls. The PR-10 family has sequence similarity to ribonucleases and is the only family consisting of cytoplasmic proteins, but there is no evidence that PR-10 proteins are active against e.g. viruses. The PR-1 and PR-5 proteins are often strongly induced and seem to affect membranes, but their precise actions have not been elucidated. The inducible PRs are mostly acidic proteins that are secreted into the intercellular space of the leaf. Both through cDNA sequence homologies and on the basis of similar enzymatic activities, additional basic counterparts have been identified. These basic PRs occur at relatively low levels in the vacuole and, besides being induced upon infection, are expressed in a tissue-specific and developmentally-controlled manner in leaves, roots and floral parts (Eyal and Fluhr, 1991, Linthorst, 1991). Specific activities vary greatly, *i.e.* from 5 nKat/mg with laminar in as a substrate for the inducible, acidic glucanase PR-2a to 23 and 1300 nKat/mg for -2b and -2c, respectively, and 1100 nKat/mg for the developmentally-controlled basic glucanase PR-2e (Kauffmann *et al.*,1987). It has been suggested that in induced plants the accumulated intercellular proteins form the first line of defense to a challenging pathogen and, if this fails and the tissue is disrupted, the release of the vacuolar PRs functions as a second line, engulfing the pathogen with lytic enzymes (Mauch and Staehelin, 1989). The constitutive expression of several of the basic proteins in older leaves, roots and developing flowers could be similarly considered as a protective mechanism against pathogen invasion, possibly contributing to the often observed increase in resistance with plant age. However, the organ-specific expression of specific PR genes suggests that the proteins also play roles in normal developmental processes.

A. Effects of pathogenesis-related proteins on expression of resistance

Constitutive expression of individual PRs in transgenic plants can lead to reduced pathogen growth and symptom expression, consistent with a role of PRs in the expression of acquired resistance (Ryals *et al.*,1994). However, such effects are by no means general and pathogens may have evolved mechanisms to reduce the effects of PRs. Thus, many chitin-containing fungi are not inhibited by chitinases, presumably because the chitin in their cell walls is shielded by a protective layer. Such a layer may be less developed at growing hyphal tips, which can be lysed (Schlumbaum *et al.*,1986).

Significant suppression of disease symptoms caused by the soil-borne fungus *Rhizoctonia solani* was demonstrated in tobacco or canola expressing a vacuolar (class I) chitinase from bean (Broglie *et al.*,1991), the basic tobacco chitinase PR-3c (Lawton *et al.*,1993, Vierheilig *et al.*,1993), tobacco or cucumber PR-8 (Lawton *et al.*,1993), or the (class II) barley chitinase (Jach *et al.*,1995), but enhanced chitinase levels caused no significant protection against nicotianae (Neuhaus *et al.*,1991, Nielsen *et al.*,1993, Zhu *et al.*,1994) or *Fusarium oxysporum* (Van den Elzen *et al.*,1993, Jongedijk *et al.*,1995). The reduction of *R. solani* in vacuole-targeted class I chitinase transformed plants is fairly unexpected, because tobacco roots constitutively express high levels of their own class I chitinase but, nevertheless, are fully susceptible. Antifungal activity of chitinases can be synergistically enhanced by β-1,3-glucanases, both in vitro and in vivo. Thus, co-expression of chitinase and glucanase genes in tobacco enhanced resistance against *C. nicotianae* (Zhu *et al.*,1994, Jach *et al.*,1995). In tomato, simultaneous expression of the basic tobacco chitinase PR-3d and glucanase PR-2e afforded substantial protection against *F. oxysporum* f. sp. lycopersici, whereas transgenic plants expressing either one of these genes were not protected (Jongedijk *et al.*,1995). Targeting the proteins to the apoplast was not more effective, indicating that tonoplast leakagemust occur sufficiently early to halt pathogen progress. Combinations of the acidic tobacco chitinase PR-3a and glucanase PR- 2b were not effective when accumulating either in the apoplast or in the vacuole, nor were combinations of an acidic glucanase with a basic chitinase, or vice versa. The situation appears largely similar for the PR-1 and -5 proteins, which have been found to possess antifungal activity against oomycetes, which lack chitin in their cell walls. Constitutive expression of PR-1a in tobacco reduced symptoms caused by *P. tabacina* (Alexander *et al.*,1993, Lawton *et al.*,1993) and *Phytophthora parasitica* f.sp. nicotianae (Alexander *et al.*,1993), but not those provoked by the non-oomycete *C. nicotianae*, or the bacterial pathogen *Pseudomonas syringae* pv. Tabaci (Alexander *et al.*,1993). The various tomato and tobacco PR-1 proteins displayed inhibitory activity on the growth of *P. infestans* in tomato leaf disc assays, with tomato PR-1c and tobacco PR- 1g being the most effective family members (Niderman *et al.*,1995). Basic PR-5 ('osmotin') likewise has antifungal activity against *P. infestans* (Vigers *et al.*,1992, Zhu *et al.*,1996), but in transgenic tobacco no delay in symptoms caused by *P. parasitica* f.sp. nicotianae was apparent (Liu et al., 1994). So far, no results have been published on suppression of pathogens in transgenic plants expressing PR-4, but basic tobacco PR-4c exhibits antifungal activity in vitro against certain fungi and under these conditions has been found to act synergistically with basic tobacco PRs -2 and -3 (Ponstein *et al.*,1994). An additional 'SAR gene' in tobacco, SAR 8.2. when expressed constitutively in transgenic tobacco, was also found to reduce disease caused by *P. parasitica*, but the protein has not been characterized (Lawton *et al.*,1993).Taken together, these observations do not indicate a significant role of the major, pathogen-inducible PRs in the enhanced resistance expressed upon challenge inoculation of plants with SAR. Additional SAR genes, comprising both minor, developmentally-controlled PRs and those encoding mRNAs for which the protein has not been identified SAR 8.2 (Ward *et al.*,1991) glycine-rich protein (Van Kan et al., 1988, Linthorst, 1991) could have more effect. However, in as far as the activities of PRs have been determined, these are directed only against fungi; PRs or similar proteins effective against bacteria or viruses have, so far, not been identified. A role of proteinase inhibitors against insect attack is well established (Ryan, 1990), but SAR is not commonly associated with enhanced protection against insects. Screening specifically for antifungal, antibacterial and antiviral activities in plants has yielded PR-like proteins with antifungal properties related to PR-families -4 (Hejgaard *et al.*, 1992) and -5 (Vigers *et al.*,1991), as well as ribosome-inactivating proteins, thionins, lectins and defensins (Linthorst, 1991). These proteins commonly occur in storage organs, such as seeds and tubers, but may also be induced in leaves following pathogen attack (Broekaert *et al.*,1995). Judging from the synergistic actions of some of these proteins when expressed together, it may be expected that when multiple SAR genes are coordinately expressed, such as in a HR, complementary actions of the resulting proteins could yield a strong anti-pathogenic potential. Moreover, when resistance responses are activated upon challenge inoculation, PRs are induced again, and more quickly and strongly than in non-induced plants. However, the apparent lack of PRs or other induced defensive proteins with activity against bacteria and viruses is difficult to reconcile with the non-specificity of acquired resistance. It is not inconceivable that, besides the conspicuous PRs, several other compounds are induced that have antipathogenic activities, but are yet to be discovered. The significance of the inducible PRs then becomes difficult to assess at present. It is intriguing that the thaumatin like PR-5 family is expressed not only in response to pathogens, but also during osmotic stress. Thus, osmotin (tobacco PR-5c) and its homolog NP24 (PR- 5a) in tomato were independently identified as being induced by salt stress (Singh et al., 1987) and infection (Stintzi *et al.*,1991). Interestingly, lipid transfer proteins can also be induced by infection, have antifungal activity, and are expressed during drought stress (Kader, 1997). Such observations support a role for PRs as stress proteins with functions that exceed their involvement in plant-pathogen interactions.

(i) Pathogenesis-related (PR) protein 1. The first PR-1 protein was discovered in 1970. Since then, a number of PR-1 proteins have been identified in *Arabidopsis, Hordeum vulgare* (barley), *Nicotiana tabacum* (tobacco), *Oryza sativa* (rice), *Piper longum* (pepper), *Solanum lycopersicum* (tomato), *Triticum* sp. (wheat) and *Zea mays* (maize) (Liu and Xue, 2006). These PR-1 having 14 to 17 kD molecular weight and mostly of basic nature. Non-expressors of Pathogenesis- Related Genes1 (NPR1) regulate systemic acquired resistance via regulation pathogenesis related 1 (PR-1) in Arabidopsis thaliana. The interaction of nucleus-localized NPR1 with TGA transcription factors, after reduction of cysteine residues of NPR 1 by salicylic acid (SA) results in the activation of defense genes of PR-1. In the absence of TAG 2 and/or SA expression of PR-1 not occur in Arabidopsis thaliana (Després *et al.*, 2000, Rochon *et al.*,2006). PR-1 proteins have antifungal activity at the micromolar level against a number of plant pathogenic fungi, including *Uromyces fabae*, *Phytophthora infestans*, and *Erysiphe graminis* (Niderman *et al.*,1995). The exact mode of action of the antifungal activities of these proteins are yet to be identified but a PR-1-like protein, helothermine, from the Mexican banded lizard have been found to be interacting with the membrane-channel proteins of target cells, inhibiting the release of Ca^{2+} (Monzingo *et al.*,1996).

(ii) â-Glucanase (PR2). Plant β -1,3-glucanases (ß-1,3-Gs)comprises of large and highly complex genefamilies involved in pathogen defense as well as a wide range of normal developmental processes. β-1,3-Gs have molecular mass in the range from 33 to 44 kDa (Hong and Meng, 2004, Saikia *et al.*,2005). These enzymes have wide range of isoelectric pH. Most of the basicâ-1,3-Gs are localized in vacuoles of the plant cells while the acidic β -1,3-Gs are secreted outside the plant cell. Wounding, hormonal signals like methyl jasmonate and ethylene (Wuand Bradford, 2003), pathogen attack like fungus *Colletotrichum lagenarium* (Ji and Ku, 2002) and some fungal elicitors releases from pathogen cell wall (Boller, 1995) can also induced â-1,3-Gs in the various parts of plant(Wu and Bradford, 2003, Saikia *et al.*,2005). The enzyme â-1,3-Gs was found to be strongly induced by ultraviolet (UV-B; 280-320 nm) radiation in primary leaves of French bean (*Phaseolus vulgaris*), so that UV-induced DNA damage is a primary step for the induction of â-1,3-Gs(Kucera *et al.*,2003). â-1,3- glucanases and chitinases are down regulated by combination of auxin and cytokinin while Abscisic acid (ABA) at a concentration of 10 µM markedly inhibited the induction of â-1,3- glucanases but not of chitinases (Rezzonico, 1998, Wu *et al.*,2001). These enzymes are found in wide variety of plants like *Arachis hypogaea* (peanut), *Cicer arietinum* (chickpea), *Nicotiana tabacum* (tobacco), etc. and having resistivity against various fungi like *Aspergillus parasiticus, A. flavs, Blumeria graminis, Colletotrichum lagenarium, Fusarium culmorum, Fusarium oxysporum, fusarium udum, Macrophomina phaseolina* and *Treptomyces sioyaensis* (Rezzonico, 1998, Wu and Bradford, 2003, Hong and Meng, 2004, Wróbel-Kwiatkowska *et al.*,2004, Liang *et al.*,2005, Roy-Barman *et al.*,2006). â-1,3- glucanases are involves in hydrolytic cleavage of the 1,3-â-D-glucosidic linkages in â-1,3- glucans, a major componant of fungi cell wall (Simmons, 1994, Høj and Fincher, 1995). So that cell lysis and cell death occur as a result of hydrolysis of glucans present in the cell wall of fungi.

(iii) Chitinases (PR3). Most of Chitinase having molecular mass in the range of 15 kDa and 43 kDa. Chitinase can be isolated from *Cicer arietinum* (chickpea) (Saikia *et al.*, 2005), *Cucumis sativus* (cucumber), *Hordeum vulgare* (barley) (Kirubakaran and Sakthivel, 2006), *Nicotiana tabacum* (tobacco) (Pu et al., 1996), *Phaseolus vulgaris* (black turtle bean) (Chu and Ng, 2005), *Solanum lycopersicum* (tomato) (Wu and Bradford, 2003) and *Vitis vinifera* (grapes) (Sluyter *et al.*,2005). Chitinases can be divided into two categories: Exochitinases, demonstrating activity only for the non-reducing end of the chitin chain; and Endochitinases, which hydrolyse internal â-1,4-glycoside bonds. Many plant endochitinases, especially those with a high isoelectric point, exhibit an additional lysozyme or lysozyme like activity (Collinge *et al.*,1993, Brunner *et al.*,1998, Schultze *et al.*,1998, Subroto *et al.*,1999). Chitinase and â-1,3-Glucanase are differentially regulated by Wounding, Methyl Jasmonate, Ethylene, and Gibberellin. Wounding and methyl jasmonate induces gene chi 9 for Chitinases expression in the tomato seeds (Wu and Bradford, 2003). In some study, it is also found that Chitinase gene are also expressed in response to stress like cold up to -2 to -5ºC (Yeh *et al.*, 2000). These Chitinases have significant antifungal activities against plant pathogenic fungi like *Alternaria* sp. For grain discoloration of rice, *Bipolaris oryzae* for brown spot of rice, *Botrytis cinerea* for blight of Tobacco, *Curvularia lunata* for leaf spot of clover, *Fusarium oxysporum, F. udum, Mycosphaerella arachidicola, Pestalotia theae* for leaf spot of tea and *Rhizoctonia solani* for sheath blight of rice (Chu and Ng 2005, Saikia *et al.*,2005, Kirubakaran and Sakthivel, 2006). The main substrate of Chitinases is chitin - a natural homopolymer of â-1,4- inked N-acetylglucosamine residues (Kasprzewska, 2003). The mode of action of PR-3 proteins is relatively simple *i.e.* Chitinases cleaves the cell wall chitin polymers in situ, resulting in a weakened cell wall and rendering fungal cells osmotically sensitive (Jach *et al.*,1995).

(iv) Chitin Binding Protein (CBP, PR4). All chitin binding proteins do not possess antifungal activities. CBP can be isolate from plant *Beta vulgaris* (sugar beat), *Hydrangea macrophylla* (hortensia), *Nicotiana tabacum* (tobacco), *Piper longum* (pepper), *Solanum lycopersicum* (tomato) and *Solanum tuberosum* (potato) and bacteria like *Streptomyces tendae* (Nielsen *et al.*,1997, Bormann *et al.*,1999, Lee *et al.,* 2001, Yangand Gong, 2002). Moleculer weight of the CBP was found to be in the range of 9 kDa to30 kDa and having basic isoelectric pH (Nielsen *et al.*,1997, Bormann *et al.*,1999,Yang and Gong, 2002). Expression of theCACBP1 chitin-binding protein isolated from cDNA library of pepper (*Capsicum annuum* L.) (CACBP1) gene was rapidly induced in the incompatible interactions upon pathogen infection, ethephon, methyl jasmonate or wounding (experimental model plant pepper). The CACBP1 gene was organ-specifically regulated in plants. High level of expression occurs in phloem of vascular bundles in leaves of pepper (Lee *et al.*,2001, Wan *et al.*,2008).CBP shows strong inhibitory effect against fungi *Aspergillus* species, *Cercosporabeticola*, *Xanthomonas campestris* and manymore and several crop fungal pathogen(Nielsen *et al.*,1997, Bormann *et al.*,1999,Lee *et al.*,2001, Yang and Gong, 2002). Enzymetically CBP has not any function but itbinds to insoluble chitin and enhances hydrolysis of chitin by other enzyme like Chitinase (Houston *et al.*,2005, Vaaje-Kolstad *et al.*,2005).

(v)Thaumatin-Like Protein (TLP, PR5). Thaumatin-like proteins comprise of polypeptides classes that share homology with thaumatin, sweet protein from *Thaumatococcus danielli* (Bennett) Benth (Cornelissen *et al.*,1986). Thaumatin-like proteins can be isolated from *Hordeum vulgare* (barley), *Actinidia deliciosa* (kiwifruit), *Zea mays* (maize), *Pseudotsuga menziesii* (douglas-firs), *Nicotiana tabacum* (tobacco), *Solanum lycopersicum* (tomato) and *Triticum* sp. (wheat) (Wurms *et al.*,1999, Fecht-Christoffers *et al.*,2003, Anand *et al.*,2004, Zamani *et al.,* 2004). Most of the TLPs have a molecular weight in the range of 18 kDa to 25 kDa and have a pH in the range from 4.5 to 5.5 (Fecht-Christoffers *et al.*,2003, Zamani *et al.*,2004). Constitutive levels of Thumatin-Like Protein is typically absent in healthy plants, with the proteins being induced exclusively in response to wounding or to pathogen attack like *Uncinula necator, Phomopsis viticola* (Monteiro *et al.*,2003). Although the specific function of many PR5 in plants is unknown, they are involved in the Acquired Systemic Resistance and in response to biotic stress, causing the inhibition of hyphal growth and reduction of spore germination, probably by a membrane permeabilization mechanism and/or by interaction with pathogen receptors (Thompson *et al.,* 2007). Linusitin is a 25-kDa Thaumatin-like Protein isolated from flax seeds. Linustin shows antifungal activity against *Alternaria alternata* by the mechanism of membrane permeabilization. Concentration of protein and lipid and composition of cell wall of fungi play a major role in these mechanisms (Anzlovar *et al.*,1998). In one study by (Menu-Bouaouiche *et al.*,2003), Thaumatin-like proteins were isolated from cherry, apple and banana shows antifungal activity against *Verticillium alboatrum* and having endo- â1,3-glucanase activity.

(vi) Proteinase inhibitors (PR-6). The peptides belonging to the PR-6 family are defined as a subclass of serine proteinase inhibitors (PIs) related to the "tomato/ potato inhibitor I" (Glazebrook, 2005) and have a typical molecular size of _8 kDa (Van Loon*et al.,* 1994) The larger group of serine PIs, which are the best studied PIs, are sub-divided, next to this PR-6 family, in tomato/potato class II PIs, Bowman-Birk PIs and Kunitz-type PIs, reviewed in (Datta and Muthukrishnan, 1999, Haq *et al.,* 2004). This proposed original definition and classification of the PR-6 family is debatable, since it is limited to a sole sub-class of serine PIs, while all types of PIs can interact with proteinases from plant-attacking organisms and could have a role in plant defense (reviewed in (Christeller and Laing, 2005, Haq *et al.*,2004). Therefore, in the literature the general term PI is often used instead of PR-6. This section also presents some examples of studies concerning PIs, which are not all strictly classified as PR-6-type proteins. PIs have the property to bind proteinases and control proteinase activity, a general function involved in many biochemical processes, and therefore could have multiple functions in planta e.g. the regulation of endogenous proteinases during seed dormancy, reserve protein mobilization; reviewed in (Haq *et al.,* 2004), apart from their proposed defensive role reviewed in (Christeller and Laing, 2005, Haq *et al.*,2004). Concerning their role in defense, PIs may act by reducing the ability of the attacker to (i) use its lytic enzymes necessary for pathogenicity for fungi (Dunaevskii, *et al.*,2005), (ii) complete its replication cycles for viruses (Gutierrez-Campos *et al.*,1999), or (iii) obtain nutrition through digestion of host proteins and thereby limit the released amount of amino acids for nematodes, insects (Urwin *et al.*,1997, Vila *et al.*,2005).Different studies have reported that PI genes can be induced upon inoculation with micro-organisms. For instance in tomato, induction of PI genes was shown upon inoculation with *Phythophtora infestans* (Peng and Black, 1976) and *Pseudomonas syringae* pv. Tomato (Pautot *et al.*,1991). (Terras *et al.*,1993) showed an in vitro antimicrobial effect, albeit relatively weak, of barley trypsin inhibitors to four important fungal plant pathogens including *Alternaria brassicicola, Ascochyta pisi, Fusarium culmorum* and *Verticillium dahliae*. Interestingly, this activity increased synergistically in combination with other PR proteins, more specifically thionins (PR-13) (Terras *et al.*,1993). As the role of specific microbial proteinases in microbial pathogenicity is not always clear, the effect of plant PIs on the activity of these enzymes has not been studied intensively.

Yet, (Dunaevskii *et al.,* 2005) showed that buckwheat trypsin inhibitors were able to inhibit in vitro proteinases of *B. cinerea*, likely to be necessary for pathogenesis (Ten Have *et al.,* 2004). Also, (Lorito *et al.*,1994) suggested that PIs could block the synthesis of chitin in fungal cell walls and consequently fungal growth, through inhibition of endogenous trypsin necessary for active chitin synthase (Machida and Saito, 1993). The in vivo effect of PIs on defense against microbial pathogens was also shown as there are reports of enhanced resistance in plants overexpressing PI genes. For instance, heterologous overexpression of *Nicotiana alata* PI genes in transgenic tobacco resulted in enhanced resistance against *B. cinerea*(Charity *et al.*,2005). Recently, it was shown that endogenous overexpression of two *A. thaliana* PI genes, including one PR-6-type gene, in transgenic A. thaliana resulted in enhanced resistance against *B. cinerea* (Chassot *et al.*,2007). Nevertheless, hitherto most of the studies on PIs have focused on their role in plant-insect interactions. It was found that PI genes are induced upon insect attack and mechanical wounding (Cordero et al., 1994, Glazebrook, 2005). The wounding, leading to activation of PI genes, is believed to mimic the chewing action of herbivorous insects. These insect and wound-induced responses have been extensively studied, especially the wound-activated systemin signaling pathway in tomato (Constabel *et al.,* 1995), and are generally linked to the octadecanoid pathway and JA signaling networks. In *A. thaliana*, insect attack or wounding activates JA and ET signaling pathways (De Vos *et al.,* 2005) giving rise to systemic induced resistance (De Vos *et al.*,2005). However, general both wounding and microbial pathogen attack activate JA signaling pathways in *A. thaliana*. Hereby, the AtMYC2 and the ethylene response (ERFs) transcription factors play essential roles to discriminate between either wounding- or microbial pathogen-related JA responses and to regulate the activation of the appropriate set of genes (Anderson et al., 2004, Lorenzo *et al.*,2004). More specifically, while AtMYC2 transcription factors activate wound responsive genes (i.e. PI genes), ERFs activate pathogen-responsive genes (i.e. the PDF gene AtPDF1.2a) (Lorenzo *et al.*,2004). Recently it could also be demonstrated that insects try to reduce wound-induced expression of PI genes in the plant, i.e. by components in their regurgitants (Lawrence *et al.,* 2007) or, by making use of a decoy mechanism which activates antagonizing signal pathways in the plant (Zarate *et al.*,2007). With respect to their anti-insect activity, PIs could act against the digestive proteases (e.g. trypsin, chymotrypsin) used by herbivorous insects. This was demonstrated in several in vitro experiments by a reduced growth rate of insects fed on artificial diets containing PIs compared to the growth rate of insects fed on diets without PIs (Harsulkar *et al.,* 1999, Markwick *et al.*,1995, Tamhane *et al.,* 2005).

The effect of PIs on insect attack in vivo was also shown as there are several reports of transgenic plants, expressing a heterologous PI gene and making them more resistant to insect attack reviewed in (Haq *et al.*,2004)). For instance, it was shown that overexpression of a PI gene from *Nicotiana alata* in transgenic apple plants inhibits the normal development of light-brown apple moth, *Epiphyas postvittana* (Maheswaran *et al.*,2007). (Duan *et al.*,1996) showed that transgenic rice plants, harboring an introduced potato PI gene, were more resistant to the pink stem borer (*Sesamia inferens*). (Vila *et al.,* 2005) demonstrated that expression of a maize PI gene in rice plants enhanced resistance against the striped stem borer (*Chilo suppressalis*). No reports were found on increased insect resistance upon overexpression of a PI gene in the plant from which they originated.

B. Characterization of plant PR-10 proteins

More than 100 PR-10 or PR-10-related sequences have been identified from various flowering plants. PR-10 proteins are identified primarily as IPR proteins, including tree pollen allergens and major food allergens (Wen *et al.*,1997). They have been reported in a variety of higher plant species of both angiosperms and gymnosperms. Among them, PR-10 proteins have been found in numerous dicots, including parsley (*Petroselinum crispum*) (Somssich *et al.*,1986) pea (*Pisum sativum*) (Fristensky *et al.*,1988) potato (*Solanum tuberosum*) (Matton and Brisson, 1989), white birch (*Betula verrucosa*) (Breiteneder *et al.*,1989, Swoboda *et al.*,1995), bean (*Phaseolus vulgaris* L.) (Walter *et al.,* 1990), soybean (Glycine max cv Mandarin) (Crowell *et al.*,1992), alder (*Alnusglutinosa*) (Breiteneder *et al.*,1992), apple (*Malus domestica*) (Vanek-Krebitz *et al.*,1995), celery (*Apiumgr aveolen*s) (Breiteneder *et al.*,1995), and alfalfa (*Medicago sativa*) (Breda*et al.*,1996, Esnault *et al.,* 1993). Among the monocots, PR-10 proteins are known to occur in asparagus (*Asparagus officinalis*)(Warner*et al.*,1992), rice (*Oryza sativa*)(Midoh and Iwata, 1996), lily (*Lilium longiflorum*) (Huang*et al.*,1997), and sorghum (*Sorghum bicolor*) (Lo*et al.*,1999). Among gymnosperms, PR-10 proteins have been found in sugar pine (*Pinus lambertiana*), eastern white pine (*Pinus strobus*), western white pine (*Pinus monticola*) (Ekramoddoullah *et al.*,1995, Yu*et al.*,2000), Douglas-fir (*Pseudotsuga menziesii*) (Ekramoddoullah *et al.*,2000), maritime pine (*Pinus pinaster*) (Dubos*et al.*,2001), and white spruce (*Picea glauca*) (Mattheus *et al.*,2003).

C. Induced expression by biotic and abiotic stresses

Most genes of the PR 10 IPR group have an open reading frame (ORF) from 456 to 489 bp encoding a polypeptide of 151-162 amino acids with molecular masses of 15-18 kDa. The inducible expression of PR-10 genes in response to attacks by pathogens has been widely investigated in a number of plant species.

Pathogens triggering a PR-10 response include viruses (Pu¨ hringer *et al.*,2000, Park *et al.*,2004, Pinto and Ricardo, 1995), bacteria(Breda *et al.*,1996, Esnault *et al.*,1993, Robert *et al.*,2001), and fungus (Swoboda *et al.*,1995, Walter *et al.*,1990, Liu *et al.*,2005, Liu *et al.*,2003). Immunocyto chemical localization has shown that the PR-10 proteins bind to the cell wall of blister rust fungus (*Cronartium ribicola*) both intracellularly and extracellularly in infected sugar pine needles (Ekramoddoullah, 2004). Immunofluorescence analysis has revealed that a Douglas-fir PR-10 protein is induced in cortical tissues of roots infected with the fungal pathogen, *Phellinus sulphurascens* Pilat. GUS activity regulated by the Ypr10*a promoter from apple (*Malus domestica*) is induced in young leaves by multiple stress factors, including pathogen attack and a fungal elicitor (Pu¨ hringer *et al.*,2000).Wound treatment usually induces PR-10 expression. As quantified by Northern blot and PR-10 promoterb- glucuronidase fusion analysis, an asparagus PR-10 gene (AoPR1) transcript is accumulated after wounding(Warner *et al.*,1992, Warner *et al.*,1994). Potato PR-10a is rapidly induced by wounding or by an elicitor, and becomes detectable 6 h after treatment, but the accumulation of PR-10c in potato is unaffected by wounding or by an elicitor (Constabel and Brisson, 1995, Despre´s *et al.*,1995). Differential expression profiles have been reported for birch PR-10a and PR-10c in response upon wounding (Poupard *et al.*,1998). In western white pine, both PR-10 mRNA transcripts and proteins are rapidly induced upon wounding(Liu *et al.*,2005, Liu *et al.*,2003). Furthermore, wounding can mimic *Cronartium ribicola* disease-activated PR-10 accumulation pattern in western white pine needles. *Cronartium ribicola* infection leads to expression of 10 PR-10 isoforms in needles, while nine of them accumulate after wounding(Liu *et al.*,2003).As an important environmental factor, cold hardiness affects the PR-10 expression. PR-10 protein accumulates to the highest level in the roots of sugar pine and western white pine in winter(Ekramoddoullah *et al.*,1995). The accumulation of PR-10 proteins by cold acclimation has also been observed in peach (Wisniewski*et al.*,2004) and mulberry (Ukaji*et al.*, 2004).The expression of PR-10 genes is also induced by other abiotic stresses, for example, by salinity in the roots of rice (Moons*et al.*,1997), by drought stress in maritime pine(Dubos *et al.*,2001) and hot pepper(Park *et al.*,2004), by dormancy in a desert legume (*Retama raetam*) (Pnueli *et al.*, 2002), by copper stress and other related oxidative stress in roots and leaves of birch (*Betula pendula*) (Koistinen *et al.*,2002, Utriainen *et al.*,1998), and by ultraviolet radiation in *Lupinus albus* L. (Pinto and Ricardo,1995).PR-10 gene expression is also regulated by plant hormones and defense-related signaling molecules, including jasmonic acid(Liu *et al.*,2003, McGee *et al.*, 2001, Moons *et al.*,1997, Rakwal *et al.*,2001, Wang *et al.*,1999), abscisic acid (ABA) (Wang *et al.*, 1999), and salicylic acid (McGee *et al.*,2001). Okadaic acid, a specific inhibitor of type 1 or type 2A serine/threonine protein phosphatase, enhances wound-induced PR-10 protein synthesis (Liu *et al.*,2003). These data suggest that defense-related signal chemicals and protein phosphorylation are involved in the signal transduction pathway leading to PR-10 activation. Based on investigation using lily anthers (Wang *et al.*,1999), have proposed that two separate signal transduction pathways are involved in the induction of PR-10 genes by ABA and methal jasmonate (MeJA), respectively.

D. Plant defensins (PR-12)

In 1990 the first plant defenses, isolated from wheat (Colilla *et al*,1990) and barley(Mendez *et al*, 1990), were originally categorized as a novel type thionin (see further) because of their similar molecular size (5 kDa) and similar number of cysteines (Boyes *et al.*,2001). However, as the structure (including the positions of the disulfide bonds)was not related to known and bthionins (Bruix*et al*,1993), they were grouped apart as g-thionins. Later, because of their antimicrobial properties and their structural similarity to mammalian and insect defensins, g-thionins were renamed "plant defensins" (Terras*et al*,1995). PDFs were taken up in the group of PR proteins and classified as the PR-12 family when Terras *et al.* (Terras *et al.*,1995) discovered two antifungal radish (*Raphanus sativus*) defensins (Rs-AFP3, Rs-AFP4) that were barely detectable in healthy uninfected leaves but accumulated at high levels after fungal infection. Hitherto, numerous PDFs could be purified from several plant species and plant tissues such as seeds, stems, roots, leaves and floral organs and show a wide range of in vitro biological activities including a-amylase activity, ion-channel blocking and antibacterial activity (reviewed by Lay and Anderson, 2005, Pellegrini and Franco, 2005, Thomma *et al.*,2002). However, their best characterized activity is the ability to inhibit the growth of a broad range of filamentous fungi and yeasts in vitro. For instance, (Terras*et al*,1993, Terras et al., 1995)described a broad spectrum antifungal activity of several plant defensins isolated from Brassicaceae species, including radish, against several filamentous fungi including *B. cinerea*, *A. brassicicola* and *F. culmorum*. The presence of inorganic salts and especially divalent cations generally antagonized the antifungal activity of plant defensins, although the cation sensitivity varied with the defensing and the fungus used (Terras *et al.*,1993). Recently, insight was gained in the mode of antifungal action of two different plant defensins from radish and dahlia (Dahlia merckii), Rs-AFP2 and Dm-AMP1, respectively (Thevissen *et al.*,2000, Thevissen *et al.*,2004). Both peptides bound on distinct sphingolipids (glucosylceramide and manosyldiinositol phosphorylceramide forRs-AFP2 and Dm-AMP1, respectively) in fungal membranes and, consequently, showed a different specificity against fungal and yeasts species, including the human pathogen *Candida albicans* (Thevissen *et al.*,2004).

The precise in vivo function of PDFs remains unclear and different roles have been attributed to them. Many PDFs are expressed abundantly *e.g.* in seed (Terras *et al*,1995) whereas others are developmentally regulated (Vanoosthuyse *et al*,2001) or induced by different abiotic and biotic stress factors, including cold (Koike *et al.*,2002), drought (Do *et al.*,2004), heavy metals (Mirouze *et al.*,2006), potassium starvation(Armengaud *et al.*,2004), or microbial pathogens (Penninckx *et al*,1996, Terras *et al*,1995, Zimmerli *et al*, 2004). The latter, in addition to their in vitro antimicrobial activity, indicates a role of PDFs in the plant defense response, which is further emphasized by enhanced disease resistance phenotypes observed in different plant species heterologously overexpressing PDFgenes. For instance, (Terras *et al.*,1995) showed that transgenic tobacco overexpressing the Rs-AFP2 gene was more resistant to the fungal pathogen *Alternaria longipes*. Another study described enhanced resistance of transgenic potato to *Verticillium dahliaeby* overexpression of an alfalfa PDF gene (Gao*et al.*,2000). So far, there are no reports of enhanced resistance by overexpression of a PDF gene in the plant from which it originated.

E. Thionins (PR-13)

Like PDFs, thionins are small (5 kDa), usually basic, cysteine-rich peptides and were originally isolated from cereals (Bohlmann*et al.*,1988). Hitherto, around 100 thionin gene sequences have been identified in 15 different plant species reviewed by(Stec, 2006). Induced expression of leaf thionins could be shown upon fungal infection in barley and *A. thaliana* (Bohlmann *et al.*, 1988, Epple *et al*,1995) and consequently they were classified as the PR-13 family of PR proteins.

The main characteristic of thionins is their broad in vitro antifungal and antibacterial activity (Bohlmann *et al.*,1988, Cammue *et al.*,1992)and like plant defensins, their antimicrobial effects lead to the permeabilization of cell membranes (Stec, 2006, Thevissen *et al*,1996). Antimicrobial activity was demonstrated against phytopathogenic bacteria see references in(Castro and Fontes, 2005), phytopathogenic fungi (Cammue *et al.*,1992, Terras *et al*,1993), yeast (Castro and Fontes, 2005), and mammalian cell lines(Carrasco *et al*,1981). Combination of thionins and LTPs resulted in synergistically antifungal activity, suggesting these proteins may cooperate in membrane binding and/or permeabilization (Molina *et al*,1993), (Stec, 2006).There are also several reports on transgenic plants expressing a thionin gene and making them more resistant to fungal or bacterial pathogen attack. For example (Chan *et al.*,2005) showed that heterologous overexpression of the *A. thaliana* Thi2.1gene in transgenic tomato made these plants more resistant to bacterial wilt and *Fusarium* wilt.

Table 1: Main properties of classified families of PR proteins.

Family	Type member	Typical size (kDa)	Properties	Proposed microbial target	Original reference
PR-1	Tobacco PR-1a	15	Antifungal	Unknown	(Antoniw*et al*,1980)
PR-2	Tobacco PR-2	30	b-1,3-Glucanase	b-1,3-Glucan	(Antoniw *et al*, 1980)
PR-3	Tobacco P, Q	25-30	Chitinase (class I,II, IV,V,VI,VI)	Chitin	(Van Loon,1982)
PR-4	Tobacco 'R'	15-20	Chitinase class I,II	Chitin	(Van Loon, 1982)
PR-5	Tobacco S	25	Thaumatin-like	Membrane	(Van Loon, 1982)
PR-6	Tomato Inhibitor I	8	Proteinase-inhibitor	–a	(Green and Ryan,1972)
PR-7	Tomato P69	75	Endoproteinase	–a	(Vera and Conejero,1988)
PR-8	Cucumber chitinase	28	Chitinase class III	Chitin	(Me´ traux*et al*,1988)
PR-9	Tobacco'lignin-formingperoxidase'	35	Peroxidase	–a	(Lagriminie*t al*,1987)
PR-10	Parsley 'PR1'	17	'Ribonuclease-like'	–a	(Somssich*et al*,1986)
PR-11	Tobacco 'class V' chitinase	40	Chitinase class I	Chitin	(Melchers*et al*,1994)
PR-12	Radish Rs-AFP3	5	Defensin	Membrane	(Terras*et al*,1995)
PR-13	*Arabidopsis*THI2.1	5	Thionin	Membrane	(Epple*et al*,1995)
PR-14	Barley LTP4	9	Lipid-transfer protein	Membrane	(Garcıa-Olmedo*et al*,1995)
PR-15	Barley OxOa (germin)	20	Oxalate oxidase	–a	(Zhang*et al*,1995)
PR-16	Barley OxOLP	20	'Oxalate oxidase-like'	–a	(Wei*et al*,1998)
PR-17	Tobacco PRp27	27	Unknown	–a	(Okushima*et al*, 2000)

Table was adjusted from http://www.bio.uu.nl/wfytopath/PR-families.htm

aNo in vitro antimicrobial activity reported.

Also, high-level expression of a hordothionin gene from barley in transgenic tobacco conferred resistance to *Pseudomonas syringae* (Carmona *et al*,1993) and enhanced resistance to bacterial diseases was described in transgenic rice plants overproducing an oat cell wall-bound thionin (Iwai *et al.*, 2002). Interestingly, (Epple *et al*,1997) described that over expression of the endogenous Thi2.1thionin gene enhanced resistance of *A. thaliana* against *Fusarium oxysporum*. The latter study reports on homologous over expression and proves in this way a direct role in defense for this thionin gene in *A. thaliana*. No studies on transgenic plants with RNAi or knockout of thionin genes and their possible implication on plant defense were found. Apart from their putative defensive function based upon these data, other in vivo functions have been proposed for thionins. There are indications for a regulatory role as thionins have thioredoxin activity and hereby could act as secondary messengers in the redox regulation on enzymes (Johnson *et al*,1987). Thionins found in seeds, could also function as storage proteins, especially as a source of sulfur (Castro and Fontes, 2005).

To be complete, the main properties of all the hitherto classified PR proteins are summarized in Table 1.

F. Lipid transfer proteins (PR-14)

Lipid transfer proteins (LTPs) are small, cationic, cysteine-rich peptides found in various plant species reviews in(Carvalho and Gomes, 2001,Kader, 1996, Kader, 1997, Yeats and Rose, 2008), including barley(Molina*et al*,1993), grapevine(Girault*et al.*,2008), wheat(Sun*et al.*,2008), A. thaliana and spinach (Segura*et al*,1993)and onion(Cammue*et al*,1995). Plant LTPs are sub-divided into two families, LTP1s and LTP2s, which present molecular masses of around 9 and 7 kDa, respectively. Apart from these differences in molecular size, the other characteristics of members of these two families, such as high pI and the pattern of four conserved disulfide bridges, are similar (Carvalho and Gomes, 2007). LTPs were named because of their ability to facilitate the transfer of phospholipids between membranes in vitro(Kader,1975). They are able to transfer various types of lipids including phosphatidylinositol, phosphatidylcholine and galactolipids (Castro and Fontes, 2005). Due to this low specificity for the lipid substrate, plant LTPs are also named "non-specific lipid transfer proteins" (Kader,1996). As LTP gene expressionwas also found to respond to infection with pathogens (García-Olmedo *et al*,1995) they were classified as PR proteins. LTPs possess a signal peptide, targeting them to the cell secretory pathway (Carvalho and Gomes, 2007). Various LTPs have been shown to be localized at the cell wall, as was demonstrated for the A. thaliana LTP1gene product (Thoma *et al.*,1993). This extra-cellular location is not a general rule as LTPs have also been found in glyoxosomes where their activity was linked to lipid catabolism (Tsuboi *et al*,1992) and in protein storage vacuoles of seeds (Carvalho *et al.*,2001).

LTPgenes are generally expressed in leaves and in flowers, and rarely in roots (Arondel *et al.*,2000). In leaves, LTPgenes are usually expressed at highlevels in young expanding leaves and hereby LTPs were suggested to play a role in the transport of monomers of cutin and found to be associated with cutin and wax assembly (Chassot *et al.*, 2007, Pyee *et al.*,1994). Though, the majority of LTPgenes seem to be expressed in flowers or flower organs (Arondel *et al*, 2000, Park *et al.*,2000, Pyee *et al.*, 1994). (Park *et al.*, 2000) showed that an LTP is necessary for pollen adherence to the stigma during pollen elongation in the *Lilium longiflorum.* Next to their inducibility upon pathogen infection (García-Olmedo *et al.*,1995, Jung *et al.*, 2003), LTPgenes are also responsive to abiotic stresses like drought, cold and salt (Jang *et al.*,2004, Jung *et al.*, 2003). Based on these findings in expression patterns, many biological activities have been suggested for LTPs (Yeats and Rose, 2008), including roles in cutin synthesis (Penninckx *et al.*,1998), b-oxidation (Tsuboi *et al.*,1992), plant defense signaling (Buhot *et al.*, 2001, Maldonado *et al.*,2002), and plant defense (Girault *et al.*,2008, Kristensen *et al.*, 2000, Molina *et al.*, 1993). Concerning their role in plant defense, various LTPs have been shown to have in vitro antimicrobial activities against fungi and bacteria (Cammue *et al.*,1995, Molina *et al.*, 1993,Wang *et al.*, 2004). This observed antimicrobial activity could result from the interaction of LTPs with biological membranes, possibly leading to membrane permeabilization (Kader, 1996). There are also several reports on transgenic overexpression of LTP genes resulting in enhanced tolerance to pathogen infection. For instance, transgenic tobacco expressing a barley LTP gene showed enhanced resistance against *Pseudomonas syringae* pv. Tabaci (Molina and Garcia-Olmedo,1997). Transgenic *A. thaliana*, overexpressing a barley LTP gene, showed enhanced resistance against Pseudomonas syringaepv. tomato and *B. cinerea* (Jung*et al.*, 2005). Concerning homologous overexpression experiments, (Chassot *et al.*, 2007) described that endogenous overexpression of three LTP-like genes in *A. thaliana* resulted in enhanced tolerance to *B. cinerea*.

CONCLUSIONS

PR proteins play important role in disease resistance, seed germination and also help the plant to adapt to the environmental stress. The increasing knowledge about the PR proteins gives better idea regarding the development and defense system of plants. Primary aspects of the gene regulation of the PR proteins are understood but the study of exact mechanism of gene regulation and receptor cascade will open new ways for the plant genetic engineering technology for crop improvement.

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