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Pathogenesis-related proteins and their genes in cereals

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Abstract

Pathogenesis-related proteins (PR-proteins) are induced in plants in response to attack by microbial or insect pests. They have been classified into several groups (PR-1 through PR-14 at present) based on their amino acid sequences and biochemical functions. Many of these proteins that have been purified from infected plants or seed extracts possess antifungal or insecticidal activity. Genes and cDNA clones for all classes of PR-proteins have been isolated from a variety of cereals. Some of these genes/cDNAs have been used to transform cereals. This review presents a summary of the PR-proteins and their genes characterized from rice, wheat, barley, sorghum and maize. Efforts to improve disease or insect resistance of these cereal plants by genetic engineering using genes for PR-proteins also are discussed. In many cases, the expression of the PR-proteins either singly or in combination appears to improve resistance to fungi or insects. In addition, chromosomal location of the PR-protein genes indicates that members of the same family of PR-protein genes or sometimes even several families of PR-protein genes often are clustered in the cereal genome, suggesting coordinate regulation. Some of these PR-protein genes map closely to quantitative traits loci. Some concerns regarding the use of genes encoding PR-proteins for genetic modification of cereals also are addressed.

Abbreviations: PR-proteins – pathogenesis-related proteins; TLP – thaumatin-like protein; ABA – abscisic acid; JA – jasmonic acid; BTH – benzothiadiazole; CpTi – cowpea trypsin inhibitor; PCR – polymerase chain reaction; CaMV – cauliflower mosaic virus; QTL – quantitative traits loci; TMV – tobacco mosaic virus

Introduction

Plant transformation is a promising tool for crop improvement because it overcomes the incompatibility barriers among crop species by allowing the introduction of specific alien genes into crop plants. Thus, transformation represents a powerful supplemental tool to conventional breeding programs that can accelerate the development of elite varieties with good agronomic characteristics. As described by Repellin et al. (2001) in this volume, transformation along with marker-assisted selection is likely to become an important biotechnological component of any major breeding program within the next two decades.

Among the 29 basic food crops, four rank at the top and account for a larger share of food production than the other 25 combined (Kung, 1993). Three are cereals (wheat, rice and maize) and the other is potato. The major cereals, wheat, rice, and maize belong to the subclass Monocotyledoneae and provide most of the caloric requirements of humans and domesticated animals worldwide. Thus, food security for the world depends critically upon our ability to increase the production of cereals and keep pace with the growth in population.

This food security currently enjoyed by most developed and developing nations of the world is always at risk because of annual losses of hundreds of millions of tons of food crops (valued at billions of U.S. dollars) from diseases and insects worldwide (Anderson and Pandya-Larch, 1999). Any approach that would significantly reduce this immense loss in the field or during

storage or transport will go a long way in alleviating the food shortage projected to occur within the next 20 years because of increasing world population and decreasing availability of land for cultivation.

Although chemical pesticides and insecticides have offered protection against crop losses until now, reluctance to rely on chemicals for stabilizing yield is increasing because of environmental and health concerns. Alternative mechanisms of pest control such as biocontrol or enhancing disease resistance of crop plants by wide crosses or by introduction of genes for disease and insect resistance by novel biotechnological approaches are being explored. These approaches have the promise of making crops resistant to specific fungal pathovars or insect biotypes. However, plants are exposed to a large assortment of pathogens and insects during any crop season. This may be the reason that plants have evolved a variety of defenses that include physical as well as chemical barriers to pathogen and pest attacks. Often the plant defenses are employed all at once irrespective of the nature of the biological stress. For example, wounding by insect feeding may require that the plant defend itself not only against the insect, but also against opportunistic pathogens that may gain entry into the plant through wound sites.

Pathogenesis-related proteins and their role in plant defense

Although some of the physical defenses such as the plant cell wall components (cellulose, lignin) and chemical defenses such as tannins and phenolics represent constitutive cellular components, other defense mechanisms involve inducible components and are deployed only when they are needed. Thus, plants synthesize a variety of compounds when exposed to biological stress (fungi, bacteria, viruses, insects, or herbivores; for reviews, see Collinge and Slusarenko, 1987 and Linthorst, 1991). The inducible defenses include the following: reactive oxygen species; phytoalexins; cell wall components (callose, glycine or hydroxyproline-rich proteins); and another group of proteins called pathogenesis-related proteins (PR-proteins). The PR-proteins were identified initially as inducible proteins in leaf extracts of tobacco plants reacting hypersensitively to tobacco mosaic virus infection (for excellent reviews, see van Loon, 1985, 1999). Five distinct bands with different electrophoretic mobilities were identified in these plants that were absent in water-inoculated plants and were referred to as PR-1 through PR-5 proteins. High resolution gels, column chromatography, and immunological reactivity of the induced proteins and finally sequencing data of PR-proteins and their genes from several different plants have led to the designation of 14 distinct groups of PR-proteins so far (Van Loon and Van Strien, 1999). The PR-proteins of different groups (and often, even within the same group) differ in molecular weight, isoelectric point, and immunological cross-reactivity. It is now well established that proteins with immunological and amino-acid-sequence similarities to tobacco PR-proteins are induced in a variety of plants belonging to diverse genera of monocotyledonous and dicotyledonous plants. The specific assortments of PR-proteins induced (even in response to the same pathogen) are host-specific and often in response to different stimuli (fungal, bacterial, or insect infestation). Their induction only in pathological situations suggested (but did not prove) a role for these proteins in plant defense. The monograph by Datta and Muthukrishnan (1999) and chapters therein on individual classes of PR-proteins (PR-1 through PR-11) may be consulted for a thorough discussion of PR-proteins and their roles in plant pathogenesis and development.

Definition of PR-proteins

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.

Biological role of PR-proteins

A major clue regarding the precise biological role of PR-proteins surfaced when the biochemical activities of the PR-2 and PR-3 classes of proteins were determined. The PR-2 proteins had an endo β-1,3-glucanase activity (Kauffmann et al., 1987), whereas the PR-3 proteins (plus PR-4, PR-8, and PR-11

proteins) were shown to have endochitinase activity (Legrand et al., 1987). Most fungi contain β-1,3-glucans or chitin in their cell walls (Bartnicki-Garcia, 1968). Thus, exposure of fungal pathogens to these hydrolytic enzymes could result in two consequences. One is the release of cell wall fragments containing oligosaccharides, which have been shown to elicit defense responses (including the induction of the phenylpropanoid pathway enzymes and PR-proteins) in a variety of plants (Mauch and Staehelin, 1989; Takeuchi et al., 1990; Ham et al., 1991). Second, exposure to lytic enzymes when the cell wall is being assembled by elongation of preexisting primers of oligo-N-acetylglucosamines and oligo-β-1,3-glucans by synthetic enzymes (chitin synthase and β-1,3-glucan synthase, respectively) could interfere with this process. In addition, high concentrations of lytic enzymes may result in dissolution of cell wall, if their substrates are exposed (instead of being buried under layers of other polymers). Testing of purified chitinases and β-1,3-glucanases for antifungal activity showed that they inhibited the growth of a variety of fungi, especially in combinations (Mauch et al., 1988). Similarly, purified PR-1 and PR-5 proteins also were shown to inhibit growth of specific fungi (Roberts and Selitrennikoff, 1990; Niderman et al., 1995). The PR-5 proteins, which also are referred to as thaumatin-like proteins (TLPs, because of their sequence similarity to the intensely sweet protein thaumatin from *Thaumatococcus danielli*; Daniell, 1852) caused leakage of cytoplasmic material from the fungi and hyphal rupture. Similarly purified PR-1 protein from tobacco and tomato inhibited growth of Oomycetous fungi (Vigers et al., 1991; Woloshuk et al., 1991). The PR-6 proteins were shown to be inhibitors of proteases (Green and Ryan, 1972). Presumably, their targets are insect or microbial proteases. The PR-7 proteins are endoproteases (Vera and Conejero, 1988). The PR-9 proteins are lignin-forming peroxidases (Reimers et al., 1992; Baga et al., 1995; Johansson and Nyman, 1996) and some PR-10 proteins have been shown to have an RNAse activity (Somssich et al., 1986; Moiseyev et al., 1994). More recently, the PR-protein classification has been extended to include other inducible proteins, namely defensins (PR-12), thionins (PR-13), and lipid transfer proteins (PR-14) (Van Loon and Van Strien, 1999). The wide range of hydrolase and inhibitory activities of PR-proteins are consistent with the notion that they have a role in defending the plant against pathogen infection either by active hydrolysis of specific fungal cellular components or by a general toxic effect on their growth environment. However, because many of the PR-proteins are encoded by more than one gene and because the PR-proteins often act coordinately, no studies have shown definitively that inactivation of a specific PR-protein results in enhanced susceptibility to a pathogen or insect.

Even though it is tempting to assume that the major role of PR-proteins is in plant defense either as a mechanism to generate signal molecules or as an active defense against the spread of the pathogen, they clearly play other roles in plant development. For example, many PR-proteins are regulated developmentally and are expressed in specific tissues (flowers, roots, stems and seeds) at well-defined points in their life cycles (See Table 1.3 of Van Loon, 1999). A chitinase is required for cell separation in yeast (Kuranda and Robbins, 1991), and a chitinase has been shown to be required for somatic embryogenesis at the globular stage in carrot (De Jong et al., 1992). β-1,3-glucanase (as a component of callase that digests callose surrounding individual microspores) plays an important role in microsporogenesis (Steiglitz, 1977). For example, untimely or complete lack of expression of β-1,3-glucanase can result in male sterility in petunia, sorghum, and soybean (Izar and Frankel, 1971; Warmke and Overman, 1972; Jin et al., 1997). Similarly, specific chitinases may act like modulators of the host range of Rhizobia because of their ability to hydrolyze (or generate) 'Nod' factors that are highly modified N-acetylglucosamine oligomers (De Jong et al., 1993; Staehelin et al., 1994).

Purification of PR-proteins and isolation of PR-protein genes

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

Table 1. PR-proteins and genes in rice

PR-protein family	Class sub-family/ enzyme activity	Name	Protein/ cDNA/gene	Tissue of expression	Induced by	Authors/reference*	GenPept accession no.
PR-1	Acidic	16.5kD JIP	P	Roots	JA	Moon et al. (1997)	
PR-1	Acidic	PBZ1	C		SA, blast fungus	Midow & Itoh	D38170
PR-1			G		Pathogen	Bhargava & Hamer	AAB49685
PR-2	Basic glucanase		P	Grain	Developmental	Akiyama et al. (1996)	
PR-2	Acidic glucanase		P	Bran	Developmental	Akiyama et al. (1997)	
PR-2	Glucanase		P	Leaves	Stress	Rakwal et al. (1999)	
PR-2	Subfamily B	Gns1	G	All tissues	Wounding	Simmons et al.	CAA41685
PR-2	Subfamily A	Gns2-Gns6	G	All tissues	Various stimuli	Romero et al.	AAD10379-83
PR-2	Subfamily C	Gns7-Gns8	G	All tissues	Various stimuli	Romero et al.	AAD10384-85
PR-2	Subfamily D	Gns9	G	All tissues	Etiolation	Romero et al.	AAD10386
PR-2	Glucanase	glu1	C	Seedling		Yun et al.	AF030166
PR-2	Glucanase	Rglu3	C	Seedling		Yun et al.	AF030167
PR-2	Glucanase	E1149	C	Panicle		Itoh et al.	BAA77783
PR-2	Glucanase	S3727, S3206	C	Shoot		Itoh et al.	BAA77784-85
PR-2	Glucanase	R0989, R0990	C	Root		Itoh et al.	BAA77786-87
PR-3	Class Ib chitinase		P	Bran	Developmental	Anuratha et al. (1992)	
PR-3	Class I chitinase		P	Leaves	Pathogen	Anuratha et al. (1996)	
PR-3	Class II chitinase		P	Leaves	Pathogen	Anuratha et al. (1996)	
PR-3	Class Ib chitinase	RCH-A, RCH-B	P	Suspension cells	Oligo (NAG)	Inui et al. (1996)	
PR-3	Class III chitinase	RCH-C	P	Suspension cells	Oligo (NAG)	Inui et al. (1996)	
PR-3	Class Ia chitinase	chi11	G		Seeds	Huang et al. (1991)	P24626
PR-3	Class Ia chitinase	RCH10	G	Roots	Elicitor	Zhu & Lamb	P25765
PR-3	Class Ia chitinase	RCC1	C	Leaves	Ethephon	Nishizawa & Hibi	383024
PR-3	Class I chitinase	2-2W	C	Seed	Developmental	Anuratha et al. (1992)	
PR-3	Class Ia chitinase	Cht-1	G	Root	Stress	Nishizawa et al.	BAA03749
PR-3	Class Ia chitinase	Cht-2	G	Root		Nishizawa et al.	BAA03750
PR-3	Class Ia chitinase	Cht-3	G	Root	Stress	Nishizawa et al.	BAA03751
PR-3	Class I chitinase	CH-6	C	Suspension cells	Elicitor	Kim et al.	744092
PR-3	Class I chitinase		C	Leaf		Yun et al.	AAA51377
PR-3	Class I chitinase	RC-7	C	Leaves	Pathogen	Anuratha et al. (1996)	AAA18585
PR-3	Class I chitinase	RC-24	G	Root	Elicitor, wounding	Xu et al.	S54806
PR-3	Class I chitinase		C	Pistil	Developmental	Takakura et al.	BAA25638
PR-3	Class I chitinase		G	Pistil	Developmental	Takei et al.	BAA33762
PR-3	Chitinase		C	Leaves	Pathogen	Yun et al.	AAB58238
PR-3	Class II chitinase	Rcht2	C	Suspension cells	Elicitor	Kim et al.	AAC37516
PR-3	Class II chitinase	Oschia2a	G			Itoh et al.	BAA31997
PR-3	Class III chitinase		C	Callus	Hormones	Nagasaki et al.	BAA23807
PR-3	Class VII chitinase	S12594	C	Shoot		Truong & Itoh	BAA19793
PR-5	TLP		P	Leaf	JA, stress	Rakwal et al. (1999)	
PR-5	TLP	pPIR2	C	Leaves	Pathogen	Reimmann & Dudler (1993)	CAA48278
PR-5	TLP	C22	C	Leaves	Pathogen	Velazhahan et al. (1998)	AAB53367
PR-5	TLP	D34	C	Leaves	Pathogen	Velazhahan et al. (1998)	AAB53368

Table 1. Continued

PR-protein family	Class sub-family/ enzyme activity	Name	Protein/ cDNA/gene	Tissue of expression	Induced by	Authors/reference*	GenPept accession no.
PR-6	Cystatin	OC-1	P	Seeds	Developmental	Abe et al. (1987)	
PR-6	Cystatin	OC-2	P	Seeds	Developmental	Abe et al. (1987)	
PR-6	Cystatin	OC-26	C	Seeds	Developmental	Abe et al. (1987)	AAA33903
PR-6	Cystatin	OC-9b	C	Seeds	Developmental	Chen et al. (1992)	
PR-6	Bowman–Birk	Ose 727A	C	Embryo	Developmental	Chen et al.	AAB68026
PR-6	Bowman–Birk	Rgpi9	G	Seedling	Developmental	Yun et al.	AAB17095
PR-9	Peroxidase	PO-C1	P	Seedlings	Pathogen	Reimers et al. (1992)	
PR-9	Peroxidase		P	Roots	JA	Moons et al. (1997)	
PR-9	Peroxidase	POX22.3	G	Leaves	Pathogen	Chittoor et al.	AAC49821
PR-9	Peroxidase	POX8.1	G	Leaves	Pathogen	Chittoor et al.	AAC49819
PR-9	Peroxidase	POX5.1	G	Leaves	Pathogen	Chittoor et al.	AAC49820
PR-10	RNAse	osdrr	P	Roots	JA	Moons et al. (1997)	
PR-10	RNase	PBZ1	C		SA, prebenazole	Midoh & Iwata	BAA07369
P14	Lipid transfer protein		C	Seed	Developmental	Lee et al.	CAA69949 AAB70538-AAB70541

* Authors/reference not followed by a year are not in the reference list. They are identified by the GenPept accession no. in the next column.

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.

Current methods for cereal transformation

Biotechnology of cereals has lagged behind that of other plants because of difficulties in plant regeneration and poor transformation efficiencies. It has become evident that cultivar specificity plays a major factor in plant transformation and regeneration. However, in the past decade success and efficiency of cereal transformation protocols has increased significantly. The purpose of this section is not to review cereal transformation but rather to highlight those methods that are currently being used to produce transgenic cereals including sorghum and barley as well as wheat maize and rice. For a more exhaustive review of transgenic cereals and biotechnology, see the chapter by Repellin et al. (2001) in this volume and Vasil (1999).

Genetic engineering with PR-protein genes

With the development of protocols for efficient transformation of the major cereals, the genes for PR-proteins became natural choices for plant transformation, because they represented the best prospect for enhancing plant resistance to diseases and insects. Resistance genes which represent upstream regulatory proteins which control the expression of PR-proteins and other defense proteins have become available only recently. Among the PR-protein genes, chitinase and β-1,3-glucanase genes were most attractive because of their strong *in vitro* antifungal activities. Transgenic tobacco and canola plants overexpressing a bean chitinase gene were found to be more resistant to *Rhizoctonia solani* infection as shown by the delayed development of disease symptoms (Broglie et al., 1991). However, similar studies with a tobacco chitinase in transgenic tobacco were not effective in increasing resistance to *Cercospora nicotianae* (Neuhas et al., 1991a). Increase in resistance to two Oomycetes was accomplished by transforming tobacco plants with a PR-1a gene (Alexander et al., 1993). Studies with transgenic tobacco plants expressing a soybean β-1,3-glucanase have improved resistance to *Alternaria alternata, Phytophthora parasitica,* and *Peronospora tabacina* (Yoshikawa et al., 1993; Lusso and Kuc,

1995). However, in many cases, expression of both chitinase and β-1,3-glucanase was needed to obtain significant resistance (Jongedijk et al., 1995; Sela Buurlage et al., 1993).

In the following sections, we describe attempts to enhance resistance of cereals to fungal pathogens and insects by transformation with genes for PR-proteins.

Rice

PR-proteins and genes

PR-proteins and PR-like proteins belonging to almost all classes have been detected in rice plants, and some have been purified from rice seed, bran, or infected plants. cDNA clones and/or genes corresponding to several of these PR-proteins also have been isolated from rice. Table 1 lists some of the initial reports of PR-proteins and their genes from rice. In many cases, each class of PR-proteins has several members encoded by separate genes and having different amino acid sequences; isoelectric points; and cellular locations (vacuole *versus* apoplastic space, for example).

Transgenic rice plants expressing PR-proteins

Chitinase genes (PR-3 family)

Rice was the first cereal to be transformed with genes for PR-proteins. Perhaps because of the ease with which it can be transformed (relative to other cereals), rice also ranks first among cereals for the number of different PR-protein genes that have been used to obtain transgenic plants. A chitinase gene, *chi11*, isolated from a rice genomic library (Huang et al., 1991) was placed under the control of a CaMV 35S promoter and used for PEG-mediated transformation of rice protoplasts (Lin et al., 1995). Several independent transgenic plants were regenerated from these transformed protoplasts using selection in medium containing hygromycin. They were analyzed for the presence of the chitinase transgene by Southern blot analysis and found to contain between one and six copies of the chitinase transgene. Many, but not all, of the transgenic plants with the chitinase transgene fragments were found to express the chitinase protein as detected by western blot analysis. The level of expression varied over a 10-fold range among individual transgenic plants, suggesting that the site of integration may have had an influence on the level of expression of the transgene. Most of the primary transgenic plants were fertile and set seeds. Progeny analysis showed near-Mendelian segregation of the chitinase transgene (and the selectable marker, *hpt*) among the T_1 progeny, indicating that most of the transgenic plants had a single site of integration of the transgenes. A good correlation also existed between presence of the intact transgene and expression of the transgenic chitinase.

Expression of the Chi-11 chitinase also was studied in different tissues of the T_1 progeny (Lin et al., 1995). The expected 35 kDa rice chitinase corresponding to the *chi11* gene was detected in all tissues tested (roots, sheath, and leaves) consistent with the lack of tissue-selectivity of the CaMV 35S promoter. Interestingly, when the transgenic plants were infected with the sheath blight pathogen, *R. solani*, the level of expression of this chitinase was elevated further. Comparison of the accumulation levels and patterns of chitinases in control (nontransgenic) plants and infected transgenic plants showed that the latter had higher levels. The induction of host plant chitinases (class I and class II chitinases of sizes 35 kDa and 28 kDa) in transgenic plants apparently was unaffected, in spite of the already elevated levels of the transgenic chitinase whose expression was constitutive.

The effect of the added expression of the transgenic chitinase Chi-11 (in addition to the pathogen-inducible chitinases) on the resistance of transgenic plants to sheath blight was investigated by challenging control and transgenic plants with *R. solani* (Lin et al., 1995). Two transgenic lines that differed in their level of expression of the Chi-11 chitinase were utilized along with nontransgenic controls. Although lesions appeared on both control and transgenic plants, the progression of the disease was considerably slower in transgenic plants; the number of lesions and the sizes of the final infected area of the sheath were less. The line with the higher level of expression of the transgenic chitinase was more resistant than the line with the lower level of chitinase expression, indicating a beneficial effect of the overexpression of chitinase on resistance to the sheath blight pathogen. This experiment was repeated on a larger scale with additional transgenic lines, and a similar beneficial effect of overexpression of the *chi11* gene on disease resistance was found (Datta et al., 1997).

Datta et al. (1999) also obtained transgenic plants expressing a pathogen-inducible rice chitinase gene, RC-7, by biolistic transformation of several elite lines of rice. Once again, several independent transgenic lines were found to express the chitinase at high levels.

Transgenic plants expressing this chitinase also were found to have a significantly higher level of resistance to sheath blight compared to controls.

Nishizawa et al. (1999) introduced two rice chitinase genes (*cht-2* and *cht-3*) into two Japonica varieties of rice, Nipponbare and Koshihikari, by *Agrobacterium*-mediated transformation and obtained 93 independently derived transgenic (hygromycin-resistant) plants. The presence of the transgenic chitinase genes was confirmed by polymerase chain reaction (PCR), and their expression in leaves was followed by Northern-blot analysis. The expression and location of the transgenic chitinase also were determined by Western blotting. Although accumulation of the product of the *cht-2* gene was strictly intracellular, the product of the *cht-3* gene was found both in the apoplastic fluid and in the extracts of leaves after removal of extracellular fluid. Because a small amount of intracellular chitinase of the same size was found even in nontransformed rice plants, the authors concluded that the product of the *cht-3* gene occurred primarily in the extracellular location. Tests of the transgenic rice plants (R_1 generation) constitutively expressing the Cht-2 and Cht-3 chitinases for resistance to the rice blast pathogen *Magneporthe grisea* showed that a vast majority of them was significantly more resistant than the control nontransformed plants as indicated by lesion numbers and sizes. However, some of the R_1 plants had a diminished resistance compared to the R_0 plants. Although the authors suggested that other physiological or stress factors might account for this difference, transgene silencing in a sub-population of the progeny was likely because the expression of the chitinase genes was under the control of an enhanced CaMV 35S promoter. This point will be discussed in a later section.

Rice chitinase genes also have been introduced into other plants with beneficial effects against some pathogens. For example, transgenic cucumber plants overexpressing the *cht-2* gene had a significantly higher resistance to gray mold (Tabei et al., 1998) compared to controls. Both appressoria formation and penetration of hyphae were affected in transgenic plants. A rice chitinase gene expressed in transgenic strawberries was shown to provide increased resistance to the powdery mildew, *Sphaerotheca humuli* (Asao et al., 1997). Similarly, introduction of a basic class I chitinase gene (RCH10) resulted in a reduction in the development of black spots in transgenic rose plants (*Rosa hybrida* L.) by 13–43% (Marchant et al., 1998). Thus, the introduction of a non-host gene also can result in a beneficial effect against pathogenic fungi.

A class I chitinase gene from barley has been introduced into an Iranian rice variety, Tarom Molaii (and into two other varieties) by a biolistic procedure under the control of a rice actin promoter. Transgenic plants and their progeny were characterized by PCR, Southern and western blot analyses and found to contain the transgene and to transmit it stably over several generations. One particular line (#827) was carried through 8 generations and to express the 36 kDa barley chitinase. This line was found to be significantly more resistant to sheath blight than control plants in greenhouse trials (Ghareyazie et al., 2000).

Thaumatin-like protein genes (PR-5 family)

The cDNA for a rice TLP (PR-5 family) isolated from *R. solani*-infected rice plants (Velazhahan et al., 1998) also has been introduced into Chinsura Boro II, IR72, and IR51500 rice plants either by protoplast-mediated or biolistic transformation by Datta et al. (1999). Several independent transformants were isolated and shown to differ in the pattern of Southern blot probed with the *tlp* probe and in the level of expression of the 23 kDa rice TLP. The level of accumulation of this protein in leaves or sheaths of some transgenic plants was estimated to be in the range of 0.25 – 0.5% of total proteins, indicating high levels of this PR-protein in these plants. When several progeny of a high-expressing plant were challenged with *R. solani*, those with high expression of TLP were significantly more resistant to the pathogen compared to those with no expression of TLP and the nontransgenic control plants. A more extensive study of several independently transformed lines expressing TLP also had a similar degree of resistance to sheath blight pathogen.

Proteinase inhibitor genes (PR-6 family)

A gene for a cowpea trypsin inhibitor (CpTi) under the control of a rice actin 1 promoter was used to obtain transgenic rice plants by PEG-mediated transformation of protoplasts of a Japonica rice, Taipei 309 (Xu et al., 1996). Some of the regenerated plants were found to be resistant to glufosinate and to express the CpTi in fairly large amounts (1.3% of total soluble protein) in leaf and stem tissues. Extracts of these plants inhibited bovine trypsin as expected. Tests of third generation plants expressing CpTi under natural conditions in fields infested with striped stem borer and pink stem borer showed that several of the transgenic lines were highly resistant to these caterpillars, whereas control

nontransgenic plants exhibited lodging and dead panicles (white heads). Thus, constitutive expression of a PR-6 protein appeared to protect the rice plants against insect infestation.

A potato proteinase inhibitor gene (pin II) with its native wound-inducible promoter and a rice actin intron were introduced into Japonica rice by a biolistic procedure, and transgenic plants with high level of expression were identified (Duan et al., 1996). The transgene was shown to be inherited stably over five generations. A bioassay for resistance to pink stem borer showed that plants homozygous for the transgene were more resistant than control plants. Using the same construct, an aromatic rice variety also was transformed to obtain expression of the pinII gene (Jain et al., 1996).

A corn cystatin gene under the control of a CaMV 35S promoter was introduced into rice plants by Irie et al. (1996). The corn cystatin was expressed in both seeds and leaves, and extracts from transgenic plants inhibited papain and cathepsin. Cystatin was isolated from transgenic rice plants and shown to inhibit gut proteases of the insect *Sitophilus zeamais*. But the transgenic plants were not tested against insects for resistance to feeding damage.

A synthetic gene for the winged bean trypsin inhibitor was introduced into rice plants by *Agrobacterium*-mediated transformation (Mochizuki et al., 1999). A vast majority of the hygromycin-resistant plants (56 out of 58) also expressed the trypsin inhibitor as determined by western blot analysis; the maximum expression levels were 1–2 ng/10 μg of total protein. Growth of rice stem borer larvae on the transgenic plants with this level of expression was reduced compared to that on controls even though larval mortality was not reduced significantly under greenhouse conditions. Extracts from transgenic plants did inhibit gut proteases from rice stem borer, indicating that the transgene was expressed to yield a functional inhibitor. In the natural environment, the slower rate of growth of larvae could result in weakened larvae that could be more susceptible to natural enemies of these insects.

A rice oryzacystatin gene has been used to obtain transgenic rice calli in which the presence and expression of the gene could be demonstrated by PCR and by Northern blot analysis. Hygromycin-resistant plants were obtained from these calli, and extracts of leaves, and seeds from these plants had higher oryzacystatin activities than those from control plants (Hosayama et al., 1994). Studying how elevated levels of the cystatin affect the growth of insects on rice grains would be interesting.

Wheat

The first PR-protein to be purified and characterized extensively from any plant was the chitinase prepared from wheat germ by Molano et al. (1979). Since then, several other PR-proteins belonging to almost all classes have been identified in wheat. As with rice, several of these are inducible upon infection by pathogens or upon insect infestation. Some PR-like proteins are regulated developmentally, whereas other members of the same group are inducible by some stress. Several cDNA clones have been isolated from mRNA isolated after infection by the pathogen. Corresponding genes also have been isolated. As with other cereals, each class of PR-proteins and genes from wheat include multiple members (Table 2).

Although only a few genes for PR proteins have been introduced into wheat, they represent a large fraction of the total number of agronomic traits introduced into wheat by genetic engineering. However, effectiveness of these genes in disease control has been mixed.

Expression of chitinase, β-glucanase and ribosome inactivating proteins in transgenic wheat

Overexpressed barley-seed class II chitinase (PR-3) appears to enhance the resistance of transgenic wheat plants to powdery mildew. Bliffeld et al. (1999) introduced the chitinase gene into Bobwhite wheat together with either a ribosome-inactivating protein (RIP) gene or a β-1,3-glucanase (PR-2) gene and recovered 15 transgenic plants. Although both the RIP and glucanase activities were selectively lost in each transgenic plant, significant chitinase protein was detected in three plants as well as their progeny. Detached leaf pieces of the progeny were used to determine resistance against *Erysiphe graminis*, the fungal pathogen causing powdery mildew. Five to six days after fungal inoculation, the two transgenic plants tested exhibited significantly lower numbers of sporulating colonies of *E. graminis* than the control leaf samples.

Chitinase genes (PR-3)

Chen et al. (1998) introduced an antifungal protein gene into wheat. They used particle bombardment to introduce a rice class I chitinase gene driven

Table 2. PR-proteins and genes in wheat

PR-protein family	Class/ subfamily/ enzyme activity	Name	Protein/ cDNA/gene	Expressed in	Induced by	Authors/reference*	GenPept accession no.
PR-1	Basic	PR-1.1	C	Leaves	Pathogen	Molina et al.	CAA07473
PR-1	Neutral	PR-1.2	C	Leaves	Pathogen	Molina et al.	CAA07474
PR-2	Basic glucanase		P	Grain	Developmental	Lai et al. (1993)	
PR-2	Subfamily B	LW2	C	Aleurone layer	Developmental	Lai et al. (1993)	CAA80493
PR-2	Subfamily D	Glc1	C	Roots	Aluminum toxicity	Cruz-Ortega et al.	AAA90953
PR-2	Glucanase		P	Leaves	Pathogen	Munch-Garthoff et al. (1997)	
PR-2	Glucanase	Clone SM289	C	Spikelet	Pathogen	Li et al. (1999)	AAD28732
PR-2	Glucanase	Clone SM638	C	Spikelet	Pathogen	Li et al. (1999)	AAD28734
PR-3	Class Ib chitinase		P	Germ	Developmental	Molano et al. (1979)	
PR-3	Chitinase		P	Leaves	Aphid infestation	Van der Westhuizen et al. (1998)	
PR-3	Class Ib chitinase	Wch1	G	Leaves	Pathogen	Liao et al.	CAA53726
PR-3	Chitinase		P	Leaves	Pathogen	Munch-Garthoff et al. (1997)	CAA53626
PR-3	Class IV chitinase	SM383	C	Spikelet	Pathogen	Li et al. (1999)	AAD28730
PR-3	Class VII chitinase	SM194	C	Spikelet	Pathogen	Li et al. (1999)	AAD28733
PR-4	Chitin-binding	wPR4-8	C	Seedling		Huh et al.	AAF02296
PR-5	TLP	Trimatin	P	Seeds	Developmental	Vigers et al. (1991)	
PR-5	TLP	pWIR232	C	Leaves	Pathogen	Rebmann et al. (1991)	CAA41283
PR-5	TLP	gbx3832	C			Mingeot et al.	CAA66278
PR-5	TLP	WAS-3	P	Suspension cells		Kuwabara et al. (1999)	
PR-6	Bowman–Birk	wali3	C	Roots	Al toxicity	Snowden et al. (1995)	AAA50848
PR-6	Bowman–Birk	wali5	C	Roots	Al toxicity	Snowden et al. (1995)	AAA50850
PR-6	Z-serpin	WZC1	C	Immature grain	Developmental	Rasmussen et al.	CAA90071
PR-6	Z-serpin	WZC1c	C	Immature grain	Developmental	Jensen & Rasmussen	CAB52709
PR-6	Z-serpin	WSZ2a	C	Immature grain	Developmental	Jensen & Rasmussen	CAB52710
PR-6	Z-serpin	WZS2	C	Immature grain	Developmental	Rasmussen	CAA72273
PR-6	Z-serpin	WZS3	C	Immature grain	Developmental	Rasmussen	CAA72274
PR-6	Trypsin-inhibitor		P	Seedlings	Pathogen, SA	Molodchenkova et al. (1998)	
PR-9	Peroxidase		P	Leaves	Aphid infestation	Van der Westhuizen et al. (1998)	
PR-9	Peroxidase		C	Roots	Development	Hertig et al.	CAA37713
PR-9	Peroxidase		C	Leaves	Pathogen	Rebmann et al. (1991)	CAA39486
PR-9	Peroxidase	Wir3	C	Seedlings	Pathogen	Schweizer et al.	CAA34211
PR-9	Peroxidase	pox1	C	Roots		Baga et al. (1995)	CAA59484
PR-9	Peroxidase	pox2	C	Roots, leaves	Pathogen (leaves)	Baga et al. (1995)	CAA59485
PR-9	Peroxidase	pox3	C	Leaves		Baga et al. (1995)	CAA59486
PR-9	Peroxidase	pox4	C	Roots		Baga et al. (1995)	CAA59487
PR-13	Alpha 1 & 2 thionin	pTTH14, 1	C	Seeds	Developmental	Castagnero et al.	CAA50003-4
PR-13	type V thionin		G			Castagnero et al.	CAA54191
PR-14	Lipid transfer protein		C			Dieryck et al.	CAA45210

* Authors/reference not followed by a year are not in the reference list. They are identified by the GenPept accession no. in the next column.

by a CaMV 35S promoter together with the maize ubiquitin promoter-driven *bar* gene, which encodes phosphinothricin acetyltransferase (PAT). Out of 17 PAT-expressing transformants recovered, only 12 expressed the chitinase gene. Unfortunately, gene expression also was silenced in progeny from these plants even though molecular analysis confirmed that the promoter-coding region of the chitinase gene was intact. Nevertheless, this report demonstrated the importance of the proper promoter selection.

Thaumatin like proteins (PR-5)

Chen et al. (1999) cotransformed Bobwhite wheat plants with the CaMV 35S promoter-rice chitinase construct and a maize ubiquitin promoter-driven TLP gene. As in the previous report, the CaMV 35S promoter-driven chitinase gene was silenced. However, the *tlp* gene was expressed in the T_0 plant as well as the progeny. One transgenic event was tested for scab resistance by infecting T_1, T_2 and T_3 spikelets with *Fusarium graminearum*, the fungal pathogen that causes scab. Results from these inoculations indicated that TLP expression could delay the onset of scab. Ten days after inoculation, 43% of the spikes on control plants were infected compared to 16% on transgenic plants. However, after 14 days, the differences between control and transgenic plants became less significant. Although expression of a rice TLP delayed the progression of scab, the disease apparently was not controlled effectively. Experiments are currently in progress to introduce combinations of PR-proteins with the expectation that they will act synergistically to control scab infection.

Proteinase inhibitors (PR-6)

Proteinase inhibitors have been introduced into wheat plants to provide tolerance to insects. Altpeter et al. (1999) introduced the gene encoding a barley trypsin inhibitor, cme, into immature wheat embyros. Thirty independent transgenic wheat lines were selected, and 16 of these expressed the trypsin inhibitor up to a level of 1.1% of total protein. Feeding studies with the Angoumois grain moth (*Sitotroga cerealella*) demonstrated a significant reduction in growth rate when compared to those reared on nontransformed control seeds. Only the early-instar larvae had an increased mortality rate. In addition, the leaf-feeding insects seemed to be uninfected by the plants expressing Cme.

Barley

PR-proteins and their genes

Barley seed proteins have been studied extensively because of their importance in malting and brewing. In studies carried out at the Carlsberg laboratory and elsewhere, several proteins were isolated and chemically characterized even before their functions were known. Several chitinases, β-1,3-glucanases, TLPs, and inhibitors have been isolated from barley grains. Three major chitinases from barley seeds have been purified (Jacobsen et al., 1990; Swegle et al., 1992) and characterized. Additional isozymes have been detected in barley seeds but they have not been purified. Kragh et al. (1993) studied the tissue specificity and induction of chitinases in barley. They found that the three major chitinases in barley seeds also were secreted by suspension cell cultures of barley cells. None of these chitinases was found in the leaves after infection with powdery mildew. A basic class II chitinase was induced upon infection indicating that in barley different chitinases have distinct functions. Seven genes for β-1,3-glucanases have been identified in barley; at least four β-1,3-glucanases have been purified and characterized, and one has been expressed in *Escherichia coli* (Hrmova and Fincher, 1993; Malehorn et al., 1993; Xu et al., 1994). Two of these genes have been shown to be expressed in healthy leaves and roots. Thus, some of the β-1,3-glucanases are PR-like proteins. However, evidence indicates that some chitinases, β-1,3-glucanases, and other PR-proteins are induced in barley in response to fungal infection. For example, Ignatius et al. (1994b) showed that several isozymes of chitinases and β-1,3-glucanases were induced in barley leaves upon powdery mildew infection. Similarly, transcripts for a β-1,3-glucanase gene, pRP2 were found to accumulate in barley, wheat, sorghum, and rice leaves upon infection with *Bipolaris sorokiniana* (Jutidamrongphan et al., 1991). Induction of a PR-1 gene, a β-1,3-glucanase gene and a Bowman–Birk type of serine proteinase inhibitor gene also was reported by Stevens et al. (1996) in barley coleoptile cells infected with *Stagonospora (Septoria) nodorum*. Table 3 lists the barley PR-proteins and their cDNA/genes reported so far.

Even though the introduction of PR-protein genes into barley has not been reported yet, a number of elegant cytological studies have been carried out using barley coleoptiles injected with exogenous chitinase after infection with the powdery mildew pathogen, *E.*

Table 3. PR-proteins and genes in barley

PR-protein family	Class/ subfamily/ enzyme activity	Name	Protein/ cDNA/gene	Expressed in	Induced by	Authors/reference*	GenPep accession no.
PR-1	Basic	pHvPR-1a	C	Leaves	Pathogen	Bryngelsson et al. (1994)	CAA52893
PR-1	Basic	pHvPR1-b	C	Leaves	Pathogen	Bryngelsson et al. (1994)	CAA894
PR-1		bpr-1	G		Pathogen	Stevens et al. (1996)	CAA88618
PR-2	Basic glucanase	G1-GIII	P	Leaves	Developmental	Hrmova et al. (1993)	
PR-2	Glucanase	BHV-G1	G	Leaves	Developmental	Qi et al.	AAC14696
PR-2	Glucanase	BHV-GIII	G	Leaves	Developmental	Wang et al.	CAA47473
PR-2	Glucanase	BHV- V	C	Roots, leaves	Developmental	Xu et al. (1994)	AAA21564
PR-2	Glucanase		G	Seedlings	Developmental	Slakeski et al.	CAB41401
PR-2	Glucanase		C	Leaves	Pathogen	Gregersen et al.	AJ271367
PR-2	Glucanase		G			Litts et al.	CAA36801
PR-2	Glucanase	HV-34	G			Rodriguez et al.	AAC39322
PR-2	Glucanase		P	Leaves	Pathogen	Jutidamrongphan et al. (1991)	
PR-2	Glucanase	GII	P	Grain	Developmental	Kragj et al. (1991)	
PR-3	Class II chitinase	Chitinase C	P	Grain	Developmental	Leah et al. (1991)	
PR-3	Class I chitinase	Chitinase K	P	Grain	Developmental	Jacobsen et al. (1990)	
PR-3	Class I chitinase	Chitinase T	P	Grain	Developmental	Jacobsen et al. (1990)	
PR-3	Class II chitinase	CH1	P	Flour	Developmental	Swegle et al. (1992)	
PR-3	Class I chitinase	CH2	P	Flour	Developmental	Swegle et al. (1992)	
PR-3	Class I chitinase	CH3	P	Flour	Developmental	Swegle et al. (1992)	
PR-3	Class 1 chitinase	clone 10	C	Aleurone	Developmental	Swegle et al. (1989) Ignatius et al. (1994a)	AAA18586
PR-3	Class I chitinase	Chi33	G		Developmental	Leah et al.	AAA56787
PR-3	Class II chitinase	Chi26	G		Developmental	Leah et al.	AAA56786
PR-3	Class II chitinase	cht2a	C		Pathogen	Bryngelsson et al. (1994)	CAA55344
PR-3	Class II chitinase	cht2b	C		Pathogen	Bryngelsson et al. (1994)	CAA55345
PR-4	Chitin binding		P	Grain, leaves	Pathogen	Hejgaard et al. (1992)	
PR-5	TLP	BP-R, BP-S	P			Hejgaard et al. (1992)	
PR-5	TLP	Hv-1	P	Leaves	Pathogen	Bryngelsson et al. (1989)	
PR-5	TLP	Barperm1	C	Seeds	Developmental	Skadsen & Herbst	AAB71680
PR-5	TLP	Barperm2	C	Seeds	Developmental	Skadsen & Herbst	AAB71681
PR-6	TLP	Bsi1	P		Pathogen	Stevens et al. (1996)	CAA88618
PR-13	Thionin		P		Pathogen	Stevens et al. (1996)	CAA88618
PR-13	Thionin	DD3,DG3, DF2,DC4, DB4	C	Leaf		Bohlman & Apel	X05590- X05587 X05576
PR-13	Thionin	pKG1940 pKG1348 pKG2872	C	Leaf		Gausing	AAA32978 AAA32977 AAA32976
PR-14	Lipid transfer protein	Cw-19	C		Pathogen	Molina & Garcia-Olmedo	CAA48623

*Authors/reference not followed by a year are not in the reference list. They are identified by the GenPept accession no. in the next column.

graminis (Toyoda et al., 1991). When the coleoptile tissue of barley was detached, fixed and treated with a bacterial chitinase (*Streptomyces griseus*), haustoria were digested rapidly, and the rate of this dissolution depended both upon the concentration of the chitinase injected and the growth stage of the haustoria. Thus, the cell walls of developing haustoria apparently have chitin as a major component. When chitinase was injected directly into powdery mildew-infected epidermal cells, haustorial primordia disappeared within 4 h, and the development of secondary hyphae was inhibited completely. If the chitinase was injected after the haustoria had matured, no visible effect on haustoria was observed but the elongation of secondary hyphae was inhibited greatly. These experiments clearly stress the importance of the concentration of chitinase just at the beginning of fungal infection when haustoria are being formed. Thus constitutive overexpression of a chitinase may be useful in delaying or preventing the onset of fungal infection.

Indirect evidence indicates that some PR-proteins might be important in determination of resistance to specific fungal pathogens. For example, a comparison of induction of β-1,3-glucanase isozymes in three different leaf scald-resistant backcross isolines of barley revealed that a specific isozyme of β-1,3-glucanase may have an important role in resistance. One backcross line, BC-200, exhibited a more rapid (1 day earlier) and higher level of induction of β-1,3-glucanase activity compared to the susceptible isoline. Northern blot analysis with β-1,3-glucanase isozyme-specific probes indicated that isozyme GII may be responsible for the increased resistance of the line BC-200 to the scald fungus (Roulin et al., 1997).

Transgenic barely plants expressing a hybrid β-1,3-1,4-glucanase

The hybrid β-1,3-1,4-glucanase is evolutionarily related to β-1,3-glucanase and is present in large amounts in barley grains. It is thought to be involved in the digestion of endosperm cell wall glucans (rather than the fungal cell-wall glucan, which is predominantly β-1,3-glucan). A codon-optimized gene encoding a thermostable form of the enzyme from bacterial sources has been introduced into barley to obtain transgenic plants with the expression of the thermostable β-1,3-1,4-glucanase in the scutellum and aleurone layer cells of germinating grains (Jensen et al., 1998).

Sorghum

PR-proteins and genes

Among the cereals, sorghum has received the least amount of attention with regard to PR-protein expression. Krishnaveni et al. (1999a) reported the purification and properties of three chitinases from sorghum seeds with molecular weights of 24, 28 and 33 kDa. As expected, they had anti-fungal activity against several chitin-containing fungi, but not against those without chitin in their cell walls. Chitinases and β-1,3-glucanases also were induced in leaves when exposed to the fungus *Fusarium moniliforme*. A comparison of seed-associated chitinases with those induced in leaves indicated that they were different. Vigers et al. (1991) reported the presence in extracts of sorghum seeds of a protein with a molecular weight of 22 kDa, which cross-reacted with antibody to zeamatin, a TLP from corn (Roberts and Selitrennikoff, 1990). N-terminal sequencing of this sorghum protein also revealed high sequence similarity to zeamatin (20 out of 22 residues were identical) and to other TLPs (Vigers et al., 1991). Induction of a β-1,3-glucanase in sorghum also has been reported in response to infection with the necrotrophic pathogen, *Bipolaris sorokiniana* (Jutidamrongphan et al., 1991). PR-1, PR-6 and PR-10 proteins also have been shown to be induced in sorghum in response to fungal infection (Stevens et al., 1996; Lo and Nicholson, 1998a, b). Table 4 lists the PR-proteins and genes reported so far from sorghum.

Transgenic sorghum plants expressing PR-proteins

Chitinase genes (PR-3)

Difficulties associated with pigment production during tissue culture and their toxic effects on callus survival and/or regeneration have limited efforts to introduce transgenes into sorghum. Following the initial reports of the introduction of marker genes into sorghum (Casas et al., 1993; Godwin and Chickwamba, 1994), PR-protein genes have been introduced into sorghum by biolistic and *Agrobacterium*-mediated transformation. Zhu et al. (1998) reported the introduction of a rice chitinase gene, *chi11* under the control of a CaMV 35S promoter. Six independent transformed plants were obtained and shown to contain the rice transgene and to express the corresponding protein constitutively in leaves (and other tissues). The transgenes were passed on to progeny plants in a Mendelian

Table 4. PR-proteins and genes in sorghum

PR-protein family	Class/ subfamily/ enzyme activity	Name	Protein/ cDNA/gene	Expressed in	Induced by	Authors/reference*	GenPept accession no.
PR-2	Glucanase		P	Leaves, sheath	Pathogen	Krishnaveni et al. (1999b)	
PR-3	Chitinase		P	Leaves, sheath	Pathogen	Krishnaveni et al. (1999b)	
PR-5	TLP		P	Seeds	Developmental	Vigers et al. (1991)	
PR-6	Cystatin		C	Seedlings	Developmental	Li et al. (1996)	CAA60634
PR-10	Peroxidase		C	Mesocotyls	Non-pathogen	Lo & Nicholsen (1998b)	AAC12661
PR-14	Lipid transfer protein	LTP-1	G			Pelese-Siebenbourg et al.	CAA50660
PR-14	Lipid transfer protein	LTP-2	G			Pelese-Siebenbourg et al.	CAA50661
PR-14	Lipid transfer protein	LTP-1	C	Seedlings		Pelese-Siebenbourg et al.	CAA50660

*Authors/reference not followed by a year are not in the reference list. They are identified by the GenPept accession no. in the next column.

fashion. Persistent constitutive expression of the transgene has been demonstrated even in the T_4 generation. However, a small proportion (about 20%) of the progeny showed evidence of silencing (Krishnaveni et al., submitted).

Homozygous progeny lines derived from the primary transgenic lines were identified in an attempt to maximize the expression level of the rice chitinase. The transgenic plants were analyzed for expression of the rice chitinase just prior to challenge with the pathogen in order to distinguish those that had undergone transgene silencing and to group plants with or without transgene expression. These two groups and another group of nontransgenic (control group) plants were inoculated (in the stalk) with a spore suspension of *Fusarium thapsinum*, the stalk rot pathogen. After 3 weeks, the stalks were cut and the lengths of the main lesion and the total lesion were measured and compared. The transgenic plants expressing the rice chitinase were significantly more resistant than the nonexpressing transgenic plants and control groups, indicating a beneficial effect of the PR-protein expression in limiting the spread of the disease.

TLP gene (PR-5)

Another PR-protein gene, namely a rice TLP (PR-5 group; Velazhahan et al., 1998) also has been introduced into sorghum (inbred C401) by *Agrobacterium*-mediated transformation (Jeoung-Mee et al., in preparation). In an attempt to avoid the problem of transgene silencing, the rice TLP coding region was linked to a maize ubiquitin-intron in these constructs. Several putative transformants were obtained after selection in medium containing bialaphos. These plants were shown to contain the selectable marker bar and the rice *tlp* genes. Several of them were found to be resistant to spraying with Liberty (active ingredient phosphinothricin) and to express the 23 kDa rice TLP at quite high levels. Analysis of the T_1 progeny indicated that the transgenes were passed on to progeny in a Mendelian fashion. These plants have not been evaluated yet for resistance to stalk rot.

The level of resistance achievable by the introduction of a single PR-protein gene with constitutive high level of expression likely will be only partial. The obvious strategy is to identify combinations of PR-protein genes that are maximally effective against specific pathogens. To accomplish this, efforts are underway in our laboratories to generate transgenic sorghum (and wheat) plants with different combinations of chitinases, β-1,3-glucanases, and TLPs and identify those combinations that yield plants with maximum resistance to fungal pathogens.

Maize

PR-proteins and genes

The search for antifungal protein in maize grains eventually led to the isolation and characterization of several PR-proteins and their genes. Two chitinases have been purified from maize, and cDNA clones for chitinases also have been isolated and sequenced (Huynh et al., 1992a; Wu et al., 1994a). A cDNA clone for a β-1,3-glucanase also has been described (Wu et al., 1994b). Zeamatin, a TLP, has been purified from maize, and cDNA clones for this protein also have been isolated (Roberts and Selitrennikoff, 1990; Huynh et al., 1992b; Malehorn et al., 1994). Table 5 lists the PR-proteins and their genes from maize.

Table 5. PR-proteins and genes in maize

PR-protein family	Class/ subfamily/ enzyme activity	Name	Protein/ cDNA/gene	Expressed in	Induced by	Authors/reference*	GenPept accession no.
PR-1			P	Root	Developmental	Gillikin et al.	A33155
PR-1		mPR1	C	Seeds	Pathogen	Casacuberta et al.	S14969
PR-1			C	Leaves	Pathogen	Morris et al.	AAC25629
PR-2	Glucanase		P	Seedlings	Pathogen	Cordero et al. (1994b)	
PR-2	Glucanase		C	Seedlings		Thomas et al.	AAC69757
PR-2	Acidic glucanase		C			Wu et al. (1994b)	AAA74320
PR-3	Class I chitinase	ChitA, ChitB	P	Seeds	Developmental	Huynh et al. (1992a)	
PR-3	Chitinase		G	Seeds	Developmental	Huynh et al. (1992a)	AAA33444
PR-3	Chitinase		G	Seeds	Developmental	Huynh et al. (1992a)	AAA33445
PR-3	Chitinase		P	Seedlings	Pathogen	Cordero et al. (1994b)	
PR-3	Class I chitinase		C	Seedlings		Wu et al. (1994a)	AAA62420
PR-3	Class I chitinase		C	Seedlings		Wu et al. (1994a)	AAA62421
PR-4	Chitin binding		P	Grains	Developmental	Hejgaard et al. (1992)	
PR-4	Chitin binding		P	Leaves	Stress	Hejgaard et al. (1992)	
PR-5	TLP	Zeamatin	P	Flour	Developmental	Roberts & Selitrennikoff (1990)	
PR-5	TLP		P	Seeds	Developmental	Huynh et al. (1992b)	
PR-5	TLP	Zlp	C	Seeds	Developmental	Malehorn et al. (1994)	AAA92882
PR-5	TLP		C	Leaves	Pathogen	Morris et al.	AAC25629
PR-6	Bowman–Birk	WIP1	C	Coleoptiles	Wounding	Rohrmeier & Lehle (1993)	CAA50519
PR-6		MPI	C	Embryos	Pathogen	Cordero et al. (1994a)	CAA55588
PR-6	Cystatin		C	Kernels	Developmental	Abe et al.	BAA01472

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We are not aware of any published work on the introduction of PR-protein genes into maize. Undoubtedly, these studies are either in progress or in the private domain and have not yet appeared in the published literature.

Mapping of PR-protein genes and transgenes in cereals

Although isolated reports have appeared, Li et al. (1999) reported extensive mapping of PR-protein genes in a cereal using diverse probes of cereal origin and Southern blot analyses of wheat aneuploids and mapping populations. The wheat aneuploids consisted of stocks in which either a whole chromosome or an arm is missing (Sears, 1966). The mapping population consisted of 114 recombinant inbred lines from a cross between the hard red spring wheat (*Triticum aestivum* L.) cultivar 'Opata 85' and a synthetic hexaploid wheat, W-784 (Nelson et al., 1995). This is an immortal mapping population developed under the auspices of the International Triticeae Mapping Initiative (ITMI) with over 1000 mapped genetic markers including several major (single gene) or minor disease-resistance loci (quantitative disease resistance or resistance QTLs) (see http:/wheat.pw.usda.gov/graingenes.html).

The cloned gene probes used in this study consisted of several classes of defense response (DR) genes and included hypersensitive response (HR) genes and PR-protein genes. Included in the PR-protein gene probes were clones for various subclasses of PR-1; PR-2 (β-glucanases); PR-3 (chitinases); PR-4 (chitin-binding proteins); PR-5 (thaumatin-like proteins); PR-6 (a wound-inducible Bowman–Birk type proteinase inhibitor from maize); and PR-9 (peroxidase). Also included were probes for genes of the flavonoid metabolic pathway (e.g. myb, chalcone isomerase and synthase, phenylalanine ammonia lyase, etc.), ion channel regulators, and other miscellaneous DR genes (e.g. lipoxygenase, polyphenol oxidase, lectin, and superoxide dismutase). The 36 cloned genes detected 322 fragments, and 167 loci were assigned to all 21 chromosomes of wheat. Both a high

degree of conservation and a rapid rate of evolution were observed among the members of the DR and PR gene families. As expected for hexaploid wheat, most loci were triplicated on homoeologous sets of chromosome, but others such as RIP were detected at only one locus on chromosome 6D. Many, such as chitinases and TLPs were present on more than one set of chromosomes. A peroxidase gene probe from maize detected genes on group 7 chromosomes, whereas a Per2 probe from rice detected a gene on group 2 chromosomes.

Genetic linkage mapping revealed additional features of DR and PR gene distribution. The different members of the same DR and PR gene families often were found clustered in tight linkage blocks. Thus, four loci of the β-glucanase gene family were linked within a region of 5.9 cm on the long arm of wheat chromosome 3B. A similar situation was observed in barley, where seven members of the β-glucanase gene family mapped in a 20 cm region on the long arm of chromosome 3H (Li et al., 1996). In barley, other glucanase genes have been mapped on chromosomes 1H and 7H. It is evident that evolution of glucanase gene family has been accompanied by duplication (by translocation or transposition between chromosomes or unequal crossing over within a chromosome) and divergence. In parallel, the members of the evolving family of genes have assumed new functions as revealed by tissue specific expression and targeting of glucanase isoenzymes to extracellular space or diverse intracellular compartments for their roles in β-glucan metabolism during wounding, senescence, microsporogenesis and pollen tube growth or the defense response (for additional references, see Li et al., 1996). Examples of PR-protein gene clustering also exist in other cereals. For example, all the genes detectable by a barley chitinase probe were localized on barley chromosome 1 (Swegle et al., 1989). Likewise, two rice chitinase genes, Cht-1 and Cht-3 both of which are stress-induced are located within 0.8 cm of each other on rice chromosome 6, whereas another chitinase gene, Cht-2, which is not induced by stress, is located on chromosome 5 (Nishizawa et al., 1999).

In other cases, different types of DR genes are clustered in the same chromosome region. A most significant cluster of DR genes, including TLP genes (loci Tha1 and 2), and a chitinase, a catalase, and the ion channel regulator gene (Grp94), is located on the long arm of chromosome 7B. Other smaller gene clusters were found on several wheat chromosome arms. Evidently, DR gene clusters represent adaptive gene complexes that evolved in response to stress. Faris et al. (1999) applied the candidate gene approach to the mapping of QTLs for disease resistance using the ITMI wheat mapping population. Here, genes potentially involved in the biochemical pathway leading to trait expression are employed as molecular markers for QTL analysis. Results revealed that DR genes encoding oxalate oxidase, peroxidase, superoxide dismutase, chitinase and TLPs mapped within previously identified resistance QTLs. Either a resistance QTL was associated with a specific DR- or PR-gene (s) or, more commonly the same DR- or PR-gene(s) were associated with several resistance QTLs.

The cluster of DR genes on 7BL was associated with adult plant resistance to leaf rust under natural infestations. The Per2-2B locus was associated with resistance to leaf rust culture pathotype used to detect Lr23. Interestingly, the linked gene loci Oxo2 (oxalate oxidase)/1433a (ion channel regulator) on 4A contributed to minor resistance QTLs against multiple fungal diseases including tan spot, leaf rust, and powdery mildew. This approach will become increasingly more powerful as additional DR- and PR-genes are mapped and analyzed for their association with resistance QTLs.

Mapping of transgenes in wheat

Recently, a large number of transgenic cereal plants produced by biolistic transformation have been reported, and some data are now available on mapping and inheritance of transgenes by Southern analysis and fluorescent *in situ* hybridization (FISH; Chen et al., 1998, 1999). Some generalizations can be made now. Many copies of the transgene often are introduced at a single locus, or sometimes at two or more loci. If two different constructs are bombarded, they also are likely to be introduced at the same locus (cotransformation). This may be due to a two-phase integration mechanism (Kohli et al., 1998). In the first phase, cointegrates may form by splicing of several plasmids. Subsequent integration may occur in such a way that no plant sequences interrupt the plasmid sequences. Alternatively, after the first integration into the plant genome, the integration sites may serve as hot spots for additional integration events some of which may result in the interspersion of plant sequences between plasmid sequences. The integration site of the transgene on a chromosome is random, but evidence is accumulating that some fragile sites or recombina-

tion hot spots may be favored (Kohli et al., 1999). In some cases, transgene integration is associated with chromosome rearrangements and multiple integration sites on a chromosome region of several megabases (Pawlowski and Somers, 1998; Svitashev et al., 2000).

In the mapping study of DR genes in wheat described above, the probes came from rice, maize, and barley. Because gene synteny among grasses is conserved, similar PR-protein gene organizations are expected in rice, maize, barley and sorghum. As the sequencing projects for the rice genome (and other cereal genomes) move forward, they should provide a great deal of information on the organization of PR-protein genes in cereal genomes.

Concerns regarding PR-protein genes in transgenic plants

The impetus for the use of PR-protein genes in plant transformation to enhance disease resistance has been the rationale that their deployment as constitutive defenses may be a better strategy than the inducible defenses of the host plant. Further, because the PR-proteins are normal constituents of plants, fears of the public about their use in genetic engineering may be allayed. In fact, many PR-proteins are fairly abundant in cereal grains. A good source of chitinase is wheat germ or barley flour (Molano et al., 1983; Swegle et al., 1992). However, a greater understanding of the role of PR-proteins and their analogs in plant development has raised some concerns (Neuhas, 1999). The major concern is the possibility of allergic reactions to humans (and animals) from PR-proteins that may end up in foods . This concern is particularly true for chitinases because several (such as those from rubber and one from avocado) are known to be allergenic (Posch et al., 1997). Overexpression of a class IV chitinase and a TLP in grapes affected wine quality (Waters et al., 1996). One way of overcoming these problems is to make the expression of the PR-proteins only in tissues where they are needed (such as green tissues) by using tissue-specific or inducible promoters that can be turned on with appropriate stimuli (chemicals or wounding for example). A choice can be made to utilize genes for those PR-proteins that are already present in large quantities in normal foods (e.g. wheat and rice chitinases or TLP's for example; Neuhas, 1999).

Some unexpected consequences of over-expression of PR-proteins may occur in some situations. For example, expression of a β-1,3-glucanase in floral organs may interfere with microsporogenesis. Mistiming of the appearance of callase (a tapetum-specific β-1,3-glucanase structurally different from the PR-2 class β-1,3-glucanase) leads to dissolution of the callose-containing tetrad walls and has been shown to result in male sterility in petunia, sorghum, and soybean (Izhar and Frankel, 1971; Warmke et al., 1972; Jin et al., 1997; Tsuchiya et al., 1995). Thus, when a basic tobacco β-1,3-glucanase gene with a deletion corresponding to the C-terminal sequence required for vacuolar targeting (and designed to cause secretion of the β-1,3-glucanase into the anther locule from the tapetum) was introduced into tobacco plants under the control of either a tapetum-specific promoter or 35S CaMV promoter, male sterility was observed with the former but not with the latter (Worral et al., 1992). Although achieving male sterility may or may not be desirable depending on the crop being cultivated, one must exercise caution in using particular promoters that lack tissue specificity.

Alterations in the level of expression of β-1,3-glucanase could result in greater or lowered susceptibility to microbial infection in some plants. For example, a β-1,3-glucanase I-deficient mutant of tobacco (Havana 425) was distinctly less susceptible to infection by tobacco mosaic virus (TMV) infection (Beffa et al., 1996). This result has been attributed to the alterations in the extent of callose deposition in and around lesions caused by TMV. Presumably, deficiency of β-1,3-glucanase led to an excessive deposition of callose at these sites in response to infection because of an imbalance in the synthesis versus degradation of callose that occurred. As indicated earlier in this review, over-expression of β-1,3-glucanase resulted in increased resistance to some fungal pathogens (Yoshikawa et al., 1993: Lusso and Kuc, 1995). Similarly, overproduction of PR-proteins in the roots might result in their secretion into the soil and inhibition of growth of beneficial microorganisms such as mycorrhizae, Rhizobia, and other microbes involved in decomposition of organic matter. For example, colonization by the mycorrhizal fungus *Glomus mosseau* in transgenic tobacco plants overexpressing PR-proteins, was not affected in most cases. But root colonization was affected in plants overexpressing a class II β-1,3-glucanase (Glandorf et al., 1997). Choosing promoters that are expressed only in green tissues might be more desirable than choosing those that allow expression in all tissues including roots.

Phytophagous insects may compensate for the presence of protease inhibitors in their diets by over-

expressing proteases or by elaborating a different type of protease (Bolter and Jongsma, 1995; Bown et al., 1997). This might lead to the selection of insects that are resistant to a specific protease inhibitor expressed in transgenic plants. One way of overcoming this problem is to expose them to a mixture of inhibitors, as is the case in nature. Inhibitors with different specificities could be deployed including those with multiple binding sites for different proteases.

Another concern about the introduction of PR-protein genes is the possible presence in these transgenic plants of the selectable marker gene. Typically, genes for resistance to antibiotics (e.g. hygromycin) or herbicides (e.g. bialaphos) are utilized for selection of transgenic plants. Even though these genes might be passed on to bacteria in the mammalian digestive tract, such exchange of DNA encoding antibiotic-resistance genes is more likely to occur between bacteria that are normal residents of the large intestine. The added risk from transgenic plants as sources of resistance genes might be minuscule. However, this point needs to be investigated thoroughly. Perhaps of greater concern is the likely possibility that the genes for the selectable markers would be transferred to weeds closely related to cereals. For example, wild crosses between Johnsongrass or shattercane and sorghum have been known to occur. Similarly, wheat might cross with goat grass. Such a horizontal transfer of genes through wind- or insect-assisted pollen dispersal might result in the generation of herbicide-resistant weeds that would reduce the effectiveness of the particular herbicide used for selection of the transgenic plant. Alternative selection protocols will need to be developed. Some of the ideas currently being tested involve the use of other selectable agents including green fluorescent protein, cyanamide resistance conferred by the *cah* gene (Maier-Greiner et al., 1991, 1994; Troy Weeks, pers. commun.) and the use of excision mechanisms that are designed to remove the selectable marker. In addition, highly efficient protocols for *Agrobacterium* transformation are being developed that will allow selection without the use of the selectable markers.

Yet another concern regarding the constitutive deployment of PR-protein genes is the extent of yield penalty imposed on the plant for defending against the pathogen. The effective concentrations of PR-proteins needed for disease control in most cases are in the order of 0.05–0.1% of total proteins. Only in rare cases (such as osmotin) do the PR-proteins accumulate to higher levels. Thus, the yield penalty may be about 1% of the total protein or less. Lowering this further might be possible if the PR-proteins are placed under the control of an inducible promoter. In this strategy, the crops will have to be sprayed with the appropriate inducer only when fungal or insect infestation is detected.

Silencing of transgenes

The use of viral promoters such as CaMV 35S in many of the early studies with cereal transformation led to the realization that silencing of the transgene is a serious problem. For example, about 25% of the progeny plants in the T_3 generation of transgenic rice expressing a chitinase gene under the control of the CaMV 35S promoter showed silencing of the transgene. The silenced phenotype was not reversed in the next generation, indicating a permanent alteration, even though no obvious changes in the transgene (including methylation of CCGG sequences in the promoter region) could be demonstrated (Chareonpornwattana et al., 1999). We observed silencing of the rice chitinase transgene in transgenic sorghum plants, to an extent similar to that in transgenic rice (Zhu et al., 1998). The situation was even less encouraging in wheat plants transformed with a rice chitinase and the CaMV 35S promoter. Even though the T_0 plants expressed the rice chitinase constitutively at high levels, the T_1 progeny plants had virtually no expression indicating that this promoter is inactivated more rapidly in wheat than in rice (Chen et al., 1998, 1999). Even the selectable marker gene *(hpt)* under the control of another CaMV 35S promoter was silenced. However, two other genes in the same transgenic plant (and their progeny) under the control of maize ubiquitin had high levels of expression of the transgenes (a rice *tlp* gene and the *bar* gene) even in the T_3 generation (Chen et al., 1999). The silenced and expressed genes were linked tightly and were located at the same locus on chromosome 6A as discussed above.

Future prospects

In the immediate future, we might expect an explosion of results on the use of combinations of PR-proteins that are most effective against specific pathogens in each of the major cereals. A substantial choice of genes/cDNAs from cereal sources is currently available, but additional ones undoubtedly will be described, some of them from wide relatives of domesticated cereals. In addition, efforts ought to be directed

at resolving the question of whether genes from non-host plants might be more effective than genes from the same or related hosts for disease control. Similarly, targeting of the most effective antifungal PR-proteins to appropriate cellular locations may also prove effective in control of pathogens (Melchers et al., 1993). Even though overexpression of PR-proteins may not result in complete resistance to a specific pathogen, the degree of protection afforded by specific combinations may be sufficient to be useful agronomically. A probable benefit of such incomplete resistance might be the delay or prevention of the development of pathogens and pests resistant to these enhanced host defenses.

Several laboratories are studying the introduction of upstream regulatory genes instead of the final array of defense protein genes. Included in this category are the genes for Pto or NPR1 or their cereal homologs, which are involved in the signal transduction pathway leading to activation of PR-protein genes (Zhou, 1999). Even though this approach seems quite attractive in the sense that only a single gene needs to be engineered, we often cannot anticipate all the defense genes that might be induced in a specific crop or the consequence of such an induction upon agronomic properties of the crop plant. In the wild, plant defenses against pests are related to survival of the species whereas farmers are concerned about the agronomic price that they must pay in order to stave off pest infestations. Thus, constitutive high-level expression of all PR-proteins may not be a desirable strategy. Selective induction of just the needed assortment of defense protein genes effective against an endemic pathogen may be preferable. An alternative strategy might be the use of inducible promoters that can be activated only when needed in parts of the field where infestation is first noted. Thus one might think of the use of salicylic acid- or benzothiadiazole-inducible promoters to drive the expression of the desired PR-protein gene.

In conclusion, the PR-protein genes are powerful defense agents that are currently available for genetic enhancement of cereals susceptible to endemic diseases and insect pests. They represent natural defenses of plants and thus are least likely to create unexpected or undesirable side effects and most likely to be accepted by consumers. Although combinations of PR-proteins may not result in complete resistance to a given disease (as resistance genes may), they are likely to offer broad range protection against a large assortment of pathogens and pests, which will make them very valuable to farmers.

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