13
Puroindolines: Their Role in Grain Hardness and Plant Defence

MICHAEL J. GIROUX,* THANYA SRIPO, SHIRLEY GERHARDT AND JOHN SHERWOOD

Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717-3150, USA

Introduction

The puroindolines are unique tryptophan-rich proteins found only in the Triticeae. There are just two proteins known as puroindolines, puroindoline a and b (Gautier *et al.*, 1994). Puroindoline A (PINA) and B (PINB) are small, cysteine rich, and hydrophobic endosperm-specific proteins that are involved in two major roles. These roles are wheat functionality and end use properties (reviewed in Morris, 2002), and seed defence against fungal pathogens. The puroindolines are important in affecting wheat end use properties because of their control of wheat grain hardness. Wheat grain hardness is an important grain trait as it affects nearly all end product quality traits. The puroindolines have also been demonstrated to have potent antifungal properties. Both of the major roles of puroindolines have been demonstrated via a combination of genetic research and plant genetic engineering, and are the subject of this review.

Puroindolines and grain hardness

Grain hardness of wheat (*Triticum aestivum* L. em Thell.) is perhaps the largest single major factor affecting wheat end product quality. Hexaploid wheat grain is classified both genetically and in world trade as soft or hard. Grain hardness is simply inherited, controlled primarily by a locus termed *Hardness* (*Ha*) (Symes, 1965; Baker, 1977) residing on the short arm of chromosome 5D (Mattern *et al.*, 1973; Law *et al.*, 1978; Campbell *et al.*, 1999, 2001). The ability to manipulate cereal grain texture has obvious important implications due to the large impact of grain texture on cereal end use quality. Endosperm texture alone controls much of the end product quality differences between soft and hard wheat. Starch granules in soft wheat flour are less

*To whom correspondence may be addressed: (mgiroux@montana.edu)

Abbreviations: PINA, puroindoline A; PINB, puroindoline B.

Biotechnology and Genetic Engineering Reviews – Vol. 20, December 2003
0264–8725/03/20/277–290 $20.00 + $0.00 © Intercept Ltd. PO Box 716, Andover, Hampshire, SP10 1YG, UK

damaged. As a result, soft wheat flours typically absorb less water in cooking, which gives a tenderer baked product. Therefore, soft wheat flour is typically used for pastry, cookies, or cakes, while hard wheat is used primarily for bread (reviewed in Morris and Rose, 1996). Thus, wheat grain hardness has direct effects on both milling and baking quality of wheat and wheat flour-based products. Starch damage, milling energy, particle size, water absorption, and protein content are all positively correlated with wheat grain hardness (Symes, 1965, 1969). Differences unrelated to protein content are largely controlled by the *Ha* locus, and in studies of the baking quality of near isogenic soft/hard lines, hard wheats were always superior to soft wheats (Symes, 1969).

Evidence for puroindoline's role in grain hardness

An extensive review of the role and history of the puroindolines was recently published (Morris, 2002); therefore, this review will discuss this topic briefly. The first evidence that puroindolines were involved in grain hardness came in a report by Greenwell and Schofield (1986). They reported a protein, termed friabilin, found in larger amounts in soft wheat starch than hard, and absent in durum wheat. Friabilin is controlled by chromosome 5D (Jolly *et al.*, 1993), suggesting a direct relationship between the component(s) of the marker protein friabilin and the *Ha* locus. Subsequent N-terminal sequencing of this marker protein indicated the presence of two proteins (Jolly *et al.*, 1993; Morris *et al.*, 1994). Comparison of the N-terminal sequences with that of the lipid binding proteins PINA and PINB made it clear that friabilin N-terminal sequences (Jolly *et al.*, 1993; Morris *et al.*, 1994) consisted of a 1:1 molar ratio of PINA and PINB.

PinA and *pinB* encode wheat endosperm-specific lipid binding proteins (Gautier *et al.*, 1994). The transcripts of *pinA* and *pinB* are controlled by chromosome 5D, and are undetectable in durum wheat (Giroux and Morris, 1997). There is an obvious linkage or identity between the *Hardness* locus, the marker protein friabilin, and *pinA* and *pinB*. All are controlled by the short arm of chromosome 5D, and no exceptions have been found for the relationship between the marker protein friabilin and grain softness. The data suggest the distinct possibility that differences in function for *Ha* reflect differences in function for the marker protein friabilin, which is composed of PINA and PINB. In fact, an RFLP linked to *Ha* was detected using a *pinA* probe (Sourdille *et al.*, 1996). However, no evidence was presented that linked functional or amino acid differences in PINA and hard textured grain. The results suggested that *Ha* and *pinA* were physically closely linked on 5DS. Structural differences in *pinA* or *pinB* are inseparably linked to hard textured grain (Giroux and Morris, 1997, 1998).

Puroindoline function

Structural differences in either PIN affecting their active site associated with hard texture may indicate that PINs directly affect grain hardness. The 'active site' of the PINs likely relates to their unique tryptophan-rich, hydrophobic domain (Gautier *et al.*, 1994). The PINs are soluble in the non-ionic detergent Triton X-114 (Blochet *et*

al., 1993). Triton X-114 solubility and high tryptophan content are both characteristics of integral membrane proteins (Bordier, 1981; Schiffer *et al.*, 1992). The tryptophan-rich region of PINA and PINB is suggestive of a role of the marker protein friabilin in binding lipids. In fact, the occurrence of membrane structural lipids follows that of friabilin. There are high amounts of these lipids on the surface of water-washed starch from soft wheats, low amounts on hard wheats, and none on durum (Greenblatt *et al.*, 1995). This result was suggestive of a role of starch granule membranes in the soft/hard endosperm difference. A role for these membranes was suggested by Barlow *et al.* (1973), who reported differences in the appearance of starch granules under the electron microscope that indicated the starch granule surface was the likely site of functional difference between soft and hard wheats. In addition, the purification of amyloplast membranes co-purifies PINA and PINB (Giroux and Morris, unpublished data). Structural predictions run on the amino acid sequence of PINA and PINB do not indicate any transmembrane domains (Jameson and Wolf, 1988; Garnier *et al.*, 1996). It is possible that the tryptophan-rich region of these proteins alone is responsible for their binding to lipids of starch granule membranes. A glycine to serine change (Gly-46 to Ser-46) in the tryptophan-rich domain of PINB is a common PINB alteration found only in hard wheats (Giroux and Morris, 1997). In fact, this PINB alteration is found in the majority of US hard wheats surveyed (Morris *et al.*, 2001). Different altered alleles are also associated with differences in grain hardness and end product quality (Giroux *et al.*, 2000; Martin *et al.*, 2001). The sequence changes found in hard wheats are interpreted as mutations, specifically as mutations affecting the function of the *Ha* locus. Probable effects of the Gly-46 to Ser-46 sequence change would be a decrease in the membrane affinity of the tryptophan-rich region. In comparison to the zero hydrophobicity of a glycine residue (Thorgeirsson *et al.*, 1996), the change to a serine would result in a negative hydrophobicity (–0.27). Their hydrophobic, tryptophan-rich domains may mediate the site-specific localization of these proteins to the starch surface. If so, the *pinB* serine alteration in this region may be expected to lessen the strength of lipid binding, and alter endosperm texture. Additional puroindoline mutations have been found. These include additional point mutations and stop codons in PINB (Lillemo and Morris, 2000; Morris *et al.*, 2001).

Puroindoline mutations may simply represent a tight linkage between *pinB* and *hardness*, as recently suggested (Turnbull *et al.*, 2000). However, analysis has demonstrated that a *pin* mutation can be found in all hard wheats examined in detail. Tests for recombination between the wild-type pin sequence and grain softness have been performed by numerous researchers (Giroux and Morris, 1997, 1998; Lillemo and Ringlund, 2002). There has been no evidence for recombination between the presence of wild-type PIN and grain softness. This result mirrored that of others, who found that either a *pinA* (Sourdille *et al.*, 1996) or *puroindoline*-like probe (Jolly *et al.*, 1996) detected RFLPs linked with grain hardness. In fact, the pin genes are likely contained within 50 kb of DNA similar to the situation in *Triticum monococcum* (Tranquilli *et al.*, 1999). No sequence alterations or alterations in protein abundance for either PINA or PINB have been found in any of the more than 20 soft varieties examined in detail. The *pinA* and *pinB Ha*-linked alterations are suggestive of a direct role for each of these genes in controlling grain softness, since all hard wheats examined contain alterations in either PINA or PINB.

Manipulation of cereal grain texture in transgenic plants with puroindolines

As discussed above, the available evidence strongly indicates that puroindolines are responsible for wheat grain softness (Giroux and Morris, 1998). However, this hypothesis is in dispute, and other *Ha*-linked genes, such as Gsp-1, have been put forward as controlling endosperm texture (Jolly *et al.*, 1993; Rahman *et al.*, 1994; Turnbull *et al.*, 2000). Some of the confusion perhaps results from the fact that friabilin itself is composed of other components in addition to the puroindolines, such as GSP-1 and an alpha amylase inhibitor (Jolly *et al.*, 1993; Morris *et al.*, 1994, reviewed in Morris, 2002). In short, while the alterations in PINA and PINB are intriguing, a direct cause-and-effect relationship had not been demonstrated for puroindolines and grain hardness. The puroindoline genes could be simply linked to the true *Ha* locus functional genes. Further, the common glycine to serine PINB sequence alteration found in most hard textured wheats (Morris *et al.*, 2001) may simply reflect a tight genetic linkage between *pinB* and hardness. We have carried out a complementation test of this putative *pinB Ha* locus mutation to address this question. The experiments involved transforming a hard wheat variety, which has the glycine to serine *pinB* sequence alteration, with the *pinB* sequence found in soft wheats. Successful complementation and restoration of grain softness would demonstrate that PINB is a functional part of the *Ha* locus. We recently demonstrated this (Beecher *et al.*, 2002) and found that expression of soft-type PINB in a hard background dramatically reduced grain hardness. This experiment involved overexpressing soft-type PINB using the wheat glutenin promoter (Blechl and Anderson, 1996). The resultant wheat seed was soft, opaque, and after milling, gave flour with poor flowability, typical of soft wheat flours (*Figure 13.1*).

We have also demonstrated the ability of the wheat puroindoline proteins to modify grain texture of rice (Krishnamurthy and Giroux, 2001). Expression of one or both PINs significantly reduced grain texture of rice. No synergistic effect was seen in transformants containing expression of both PINs. Grain hardness was reduced proportional to the total level of PIN in each transgenic line. The lack of a synergistic effect may indicate that PINA and PINB act independently to affect grain hardness. Rice, like maize and sorghum, does not contain any native puroindoline homologues (Gautier *et al.* 2000). Therefore, the results predict that puroindolines could be used effectively to alter grain texture of other cereals as well. In the study of pinB-transformed wheat, we confirmed the results of Barlow *et al.* (1973), who suggested a role for starch granule membrane lipids in endosperm texture. They had reported that the granule surface was the likely site of functional difference between soft and hard wheats. This was based on a rough appearance of hard wheat starch granules, whereas soft wheat starch granules have an intact-appearing amyloplast membrane having little adhering proteinaceous material. Our studies of isogenic hard/soft wheats created using transformation determined that PINB alone is able to cause the change in starch granule appearance associated with soft and hard wheat (Beecher *et al.*, 2002). The starch granules of unmodified HiLine have little associated friabilin and the rough appearance caused by adhering remnants of the proteinaceous material of the endosperm cell. In contrast, HiLine that expresses the wild-type PINB-D1a has high levels of starch-associated friabilin and smooth starch granules. This correlation of wild-type PINB's ability to interact with the surface of the starch granules as

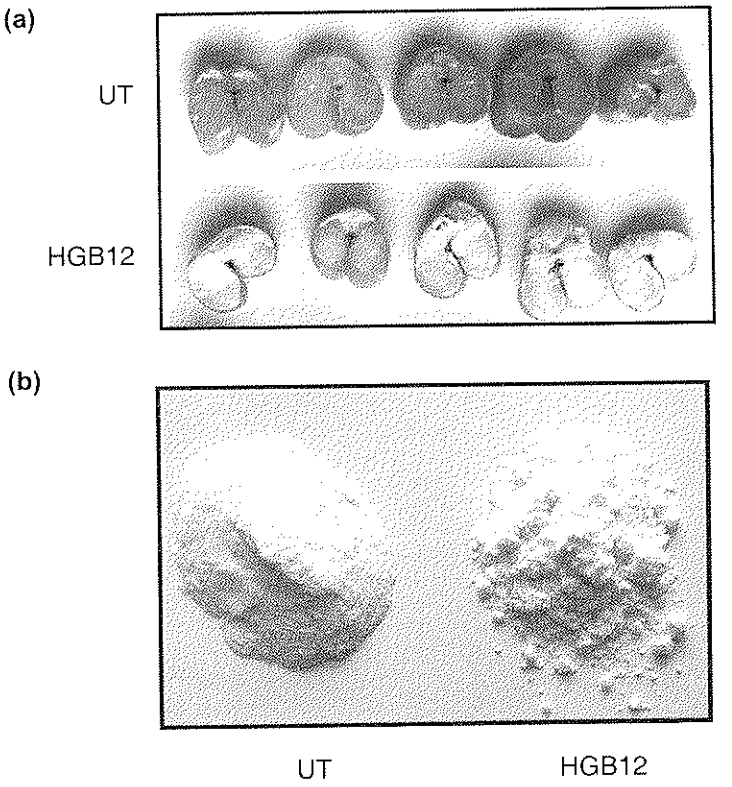

Figure 13.1. Seeds and flour of untransformed HiLine control and *pinB*-expressing transformant. (a) Cross-sections of field-grown seeds of untransformed HiLine (UT) and *pinB*-expressing transformant (HGB12). The interior of seeds of HiLine are vitreous in appearance, typical of hard red spring wheat. The interior of seeds of HGB12 HiLine expressing wild-type pinB is opaque and chalky in appearance, typical of soft wheats. (b) Straight grade white flour prepared from the hard wheat variety HiLine (UT) and HGB12. The flour of HiLine is a smooth, flowing flour typical of a hard wheat, whereas HGB12 flour is fine in particle size and coarse in appearance.

friabilin and cause smooth granule appearance and kernel softness presents a clear picture. All of these results are consistent with the hypothesis that the puroindolines control grain softness directly by reducing the interaction between starch granules and their surrounding protein matrix.

Isolation of PINA and PINB by TX114 phase partitioning and separation using gradient SDS-PAGE

The puroindolines can be enriched in concentration by a Triton X-114 non-ionic detergent phase partitioning method (Bordier, 1981). We have adapted this method for small samples and to separate PINA and PINB on SDS-PAGE (*Figure 13.2*).

PINA and PINB can be isolated from a small amount of wheat flour by a modification of the Triton X-114 phase partitioning method (Bordier, 1981). The

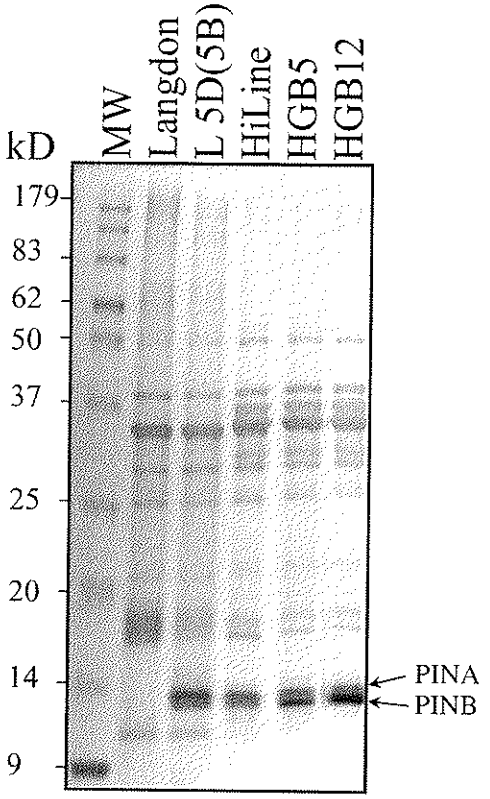

Figure 13.2. Separation of PINA and PINB in wheat and PINB expressing transformants. Arrows indicate position of PINA and PINB. Langdon is a durum variety, which has no PINA or PINB. L 5D(5B) is a substitution line of Langdon having the 5D chromosome of Chinese Spring (Langdon-CSDS5D(5B)) substituted for its 5B chromosome. HiLine is an untransformed control wheat variety having a wild-type PINA and a mutated PINB. HGB5 and HGB12 are separate transgenic lines created using the variety HiLine with added PINB.

method in use is as follows: weigh out 100 mg of finely ground kernels or flour into a 2 ml microfuge tube. Add 1 ml of Tris-buffered saline (TBS, 10 mM Tris/150 mM NaCl, pH 7.5) and 0.15 ml of 12% Triton X-114 (in TBS), and vortex samples until flour is suspended. Samples are mixed at least one hour at 4°C and then centrifuged 1 min at 13 000 × ***g*** at 4°C. The supernatant is then transferred to a fresh 1.5 ml tube and 0.5 ml of cold TBS is added to the pellet, and tubes are vortexed until pellets are suspended. Samples are then centrifuged 1 min at 13 000 × ***g*** at 4°C, and the supernatants are combined. The samples are then incubated at 37°C for 45 min, and then centrifuged 2 min at 13 000 × ***g*** at RT. The upper non-detergent phase is then aspirated off and discarded. A portion (40:1) of the remaining detergent-rich phase is transferred into a fresh 1.5 ml tube, avoiding the pellet that consists of non-TX114 soluble starch and protein contaminants. To the detergent-rich phase, 1 ml cold TBS is added, and the sample is vortexed. Tubes are placed at 37°C for 30 min, and centrifuged at 13 000 × ***g*** for 2 min at RT. The upper non-detergent phase is discarded,

and 1 ml of acetone (–20°C) is added to the detergent phase. Samples are vortexed and centrifuged at 13 000 × *g* for 5 min at 4°C, and the supernatant is discarded. TX114 soluble proteins are sequentially washed with 1 ml of acetone (–20°C) and ethyl ether (–20°C) and allowed to air dry. For gel fractionation 60:1 of SDS sample buffer w/o BME or other reducing agents is added. Prior to loading of gels, samples are incubated at 70°C for 10 minutes, with occasional mixing. The correct load is roughly 6:1 or 2.7 mg flour equivalents/lane on a 10–20% Tris–HCl gradient gel (BIO-RAD). The running conditions for the gel are as suggested by the supplier: namely, stacking at 25 mA/gel for 30 min, and separating at 35 mA/gel for 2 h 30 min. Gels are stained in Coomassie staining solution; 0.1% Coomassie blue R250, 30% methanol, and 10% acetic acid, and de-stained in 30% methanol and 10% acetic acid.

Usefulness of the puroindolines as transgenes

Although wheat in world trade is defined as soft or hard textured, this is not the case for corn, rice, and sorghum. Corn, rice, and sorghum do not appear to have *pinA* and *pinB* homologues (Gautier *et al.*, 2000), and are all hard textured, although softer textured *opaque-2* type variants exist in corn and sorghum. These softer textured variants, while having desirable nutritional properties such as increased lysine content, have increased susceptibility to pathogen contamination, and are often lower yielding. Perhaps most important, soft textured wheat does not have increased susceptibility to pathogen infection. Soft texture and lack of pathogen susceptibility both may reflect expression of PINA and PINB, since both PINA and PINB have antimicrobial properties (Dubreil *et al.*, 1998). In fact, we have strong evidence that expression of puroindolines in rice confers enhanced resistance to fungal pathogens (Krishnamurthy *et al.*, 2001). The primary effects of softer texture or smaller particle size in other cereal crops would be enhanced digestibility, reduced milling costs, and increased value. For example, in cows, more finely ground corn decreased body condition loss and milk fat, and increased milk protein and yield (Knowlton *et al.*, 1996). Similarly, reducing the particle size in corn increased gain/feed in pigs by 8% and digestibility by 7% (Wondra *et al.*, 1995).

Antifungal activity of puroindolines

IN VITRO ANTIFUNGAL ACTIVITY

As the relationship between the puroindolines and grain quality was being described, the first report that the puroindoline proteins possessed antifungal activity was also published (Dubreil *et al.*, 1998). Highly purified PINA and PINB reduced growth in broth nutrient medium of several fungi, including *Alternaria brassicola*, *Ascochyta pisi*, *Fusarium culmorum* and *Verticillium dahliae*. Fungal growth was monitored using spectrophotometry. *Botrytis cinerea* was not appreciably sensitive to either PINA or PINB. PINB was uniformly 2–3-fold more active than PINA against sensitive fungi. It was reported that the antifungal activity, when both PINA and PINB were added, was synergistic.

In vitro growth inhibition of several other fungi has also been demonstrated with puroindoline protein extracted from seeds (Balconi and Sherwood, 2001; Gerhardt *et*

al., 2002). In each case, puroindolines were extracted from Langdon durum wheat and Langdon 5D (5B), a chromosome substitution line that contains chromosome 5D from the hexaploid wheat variety Chinese Spring (Giroux and Morris, 1997). Chinese Spring chromosome 5D contains both *pinA* and *pinB*. PINA and PINB were extracted and partially purified using TX114, as described above. The extraction protocol did not separate PINA from PINB. Fungal growth inhibition was compared to growth in the presence of the Langdon extract, to Langdon 5D (5B). Fungi with a yeast-like growth habit, such as *Saccharomyces cerevisiae*, *Candida albicans*, *Ustilago hordei*, and *Cryptococcus neoformans*, were grown in broth cultures and growth measured by microscopic cell counts (Balconi and Sherwood, 2001). All showed inhibition of growth, although *U. hordei* was the most sensitive, being five times more sensitive than *S. cerevisiae*. Filamentous fungi, including *Fusarium culmorum*, *Fusarium graminearum* (Gerhardt *et al.*, 2002), *Magnaporthe grisea* and *Rhizoctonia solani* (Balconi and Sherwood, 2001), were grown on nutrient agar medium on which known amounts of the –PIN and +PIN seed extracts were spread. A small plug of the fungus was used to inoculate the centre of the plate with the test organism, and colony diameters were measured over time. Growth inhibition ranged from 20% after 4 days incubation with *F. culmorum* to 40% inhibition of growth of *M. grisea* after 7 days.

When observed under the microscope, *U. hordei* and *S. cerevisiae* cells exposed to growth-inhibiting levels of PIN were found to be misshaped and non-refractile (Balconi and Sherwood, 2001). Often, refractile organelles could be observed in the cells. The presence of this cell morphology paralleled the concentration of PIN and lack of cell growth. In cultures in which growth was delayed or slowed, a mixture of normal and altered cell morphology was observed.

Krishnamurthy *et al.* (2001) have demonstrated antifungal activity of a crude extract, as described by Blochet *et al.* (1993), from leaves of transgenic rice expressing *pinA*, *pinB*, or both *pinA* and *pinB*. These genes were fused to the maize ubiquitin promoter so that they were expressed constitutively throughout the plant. Growth of *Magnaporthe grisea*, the cause of rice blast, and *Rhizoctonia solani*, which causes rice sheath blight, was quantified by measuring colony diameter on agar amended with the PIN-containing leaf extract compared with extract from wild-type rice leaves. Growth of *M. grisea* was inhibited by approximately 35% by the extract from all transgenic plants, while growth of *R. solani* was inhibited by 41–49% by the different transgenic leaf extracts. While Dubreil *et al.* (1998) described a synergistic effect in the inhibitory activity of PINA with PINB, this was not observed by Krishnamurthy *et al.* (2001), although in the latter paper, the PIN proteins were not highly purified and PINA was not separated from PINB.

IN VIVO ANTIFUNGAL ACTIVITY

Why do wheat seeds normally have puroindoline proteins?

From an evolutionary standpoint, an obvious hypothesis is that the puroindolines protect the seed from pathogen attack during the time after the seed develops to when it germinates. A wheat seed is dormant, and therefore defenceless during that time. To test that hypothesis, the correlation between the presence and level of the puroindolines and dry seed decay was examined. Dry seed decay is caused by *Penicillium* spp. when

seed is planted into dry soil. While there may not be sufficient moisture for seed germination, there is adequate moisture for the fungus to colonize the seed, which ultimately can prevent germination. Surface-sterilized seed was placed on moistened filter paper in Petri dishes and inoculated with spores of a *Penicillium* sp. known to cause seed decay. The presence of puroindolines in Langdon 5D (5B) protected the endosperm of Langdon durum wheat that normally has no *pin* genes and transgenic HiLine that overexpresses *pinB* (data not shown). Expression of the *pin* genes in the embryo, which occurs in transgenic wheat in which the puroindoline genes are fused to the maize ubiquitin promoter, protected both the endosperm and embryo. In all cases, varieties with higher levels of *pin* expression had higher germination rates, despite inoculation with the *Penicillium* sp. Clearly, the puroindolines have a natural protective capacity for this disease. Since metabolically inactive seeds have no active defences, the presence of pre-formed puroindoline proteins would be one of the seed's few mechanisms to inhibit fungal attack.

Increased fungal disease tolerance in transgenic plants

Rice normally contains no homologues of the *pin* genes. Constitutive expression of the puroindoline genes throughout the plant was achieved by transformation of rice with the *pin* genes fused to the maize ubiquitin promoter (Krishnamurthy and Giroux, 2001). Plants were found that were successfully transformed with *pinA* (PinA+B–), *pinB* (PinA–B+) or both (PinA+B+). Others were stably transformed with the selectable marker, but neither of the *pin* genes (PinA–B–). The original purpose for creating these transformants was to demonstrate, successfully, that the expression of puroindolines in rice seed would reduce seed hardness (Krishnamurthy and Giroux, 2001). However, the availability of these plants made it possible to test for the control of fungal pathogens by the puroindolines.

When the transgenic rice were tested for reaction to rice blast, caused by *Magnaporthe grisea*, disease reduction ranged from 29% in PinA–B+ to 53% in PinA+B+, compared to the wild-type rice line M202, or PinA–B–. Similarly, when inoculated with *Rhizoctonia solani*, the cause of sheath blight, disease control ranged from 10% with PinA–B+ to 22% with PinA+B+ (Krishnamurthy *et al.*, 2001). These differences were statistically significant. In both cases, the higher the level of expression of either *pinA* or *pinB* in the leaves of the transgenic plants, as determined by Northern blots and ELISAs, the greater the disease control. When both genes were present, control appeared to be additive in terms of gene expression, rather than synergistic.

The wheat cultivar HiLine has also been transformed with *pinB* with the maize ubiquitin promoter, which would be expressed throughout the plant. Transgenic line 82, which was transformed with the ubiquitin promoter-driven construct, has been challenged with a number of fungal pathogens (*Table 13.1*). This line showed increased tolerance, compared to wild-type HiLine, or a line transformed only with the selectable marker *Bar* gene, against almost all fungal pathogens tested to date, with *Gaeumannomyces graminis*, the cause of take-all, the major exception. The transgenic wheat were also inoculated for scab (Chen *et al.*, 1999) with a Montana isolate of *F. culmorum* in the greenhouse. Disease was scored by visual inspection of the heads (Stack and McMullen, 1998). The majority of HiLine plants had between

Table 13.1. Control of wheat root rot diseases in HiLine transformed with the puroindoline B gene

Disease/pathogen	Cultivar [1]	% Disease [2]	% Disease inhibition
Fusarium root rot/*F. culmorum*	HiLine	87 ± 1.5	–
	82	42 ± 4.5	52 ± 4.4
	BAR	84 ± 1.0	3.0 ± 0.5
Rhizoctonia root rot/*R. solani*	HiLine	95 ± 2.0	–
	82	57 ± 14	38 ± 13
	BAR	79 ± 1.0	13 ± 3.5
Crown root rot/*Cochliobolis sativus*	HiLine	90 ± 3.0	–
	82	46 ± 4.5	52 ± 5.4
	BAR	79 ± 4.5	13 ± 2.1
Take-all/*Gaeumannomyces graminis*	HiLine	73 ± 10.0	–
	82	70 ± 20.0	0
	BAR	70 ± 10.0	0
Strawbreaker/*Tapesia yallundii*	HiLine	27 ± 0.5	–
	82	3.4 ± 3.3	89 ± 11
	BAR	28 ± 2.5	0
Strawbreaker/*Tapesia acuformа*	HiLine	18 ± 7.5	–
	82	0	100
	BAR	16 ± 9.0	8.5 ± 6.5

[1] Varieties are wild-type HiLine; 82, which is HiLine transformed with *pinB* using the ubiquitin promoter so that the gene is expressed throughout the plant; and BAR, which is HiLine transformed with the selectable marker *bar*, but not with *pin*.
[2] Mean and standard deviation of two replicates of 18–30 plants each. Total disease includes symptoms on a scale of 0–5.

40 and 70% infected spikelets (*Figure 13.3*). The transformant control that expressed only the *Bar* resistance gene was very similar. Line 82 showed a large increase in plants with only 0–20% infected spikelets and decreases in both the moderately and severely infected heads when compared to either control. This greenhouse experiment has been repeated with similar results (not shown).

What is the basis for the antifungal activity of the puroindolines?

The antimicrobial properties of the puroindolines may result from the inherent hydrophobicity of these proteins. PINA and PINB are among a unique group of plant proteins that have a tryptophan-rich, hydrophobic domain (Gautier *et al.*, 1994), and are soluble in the non-ionic detergent Triton X-114, a characteristic that is taken advantage of in the purification of the puroindolines (Blochet *et al.*, 1993). Triton X-114 solubility and high tryptophan content are both characteristics of integral membrane proteins (Bordier, 1981; Schiffer *et al.*, 1992) and of membrane-toxic proteins (Gatineau *et al.*, 1987). The presence of a hydrophobic tryptophan-rich region in both PINA and PINB suggests that each of these proteins strongly binds lipids, and are possibly membrane associated. Structural predictions run on the amino acid sequence of PINA and PINB indicate no likely transmembrane domains (Jameson and Wolf, 1988; Garnier *et al.*, 1996). It is possible that the tryptophan-rich region of these proteins alone is responsible for their binding to lipids and starch granule

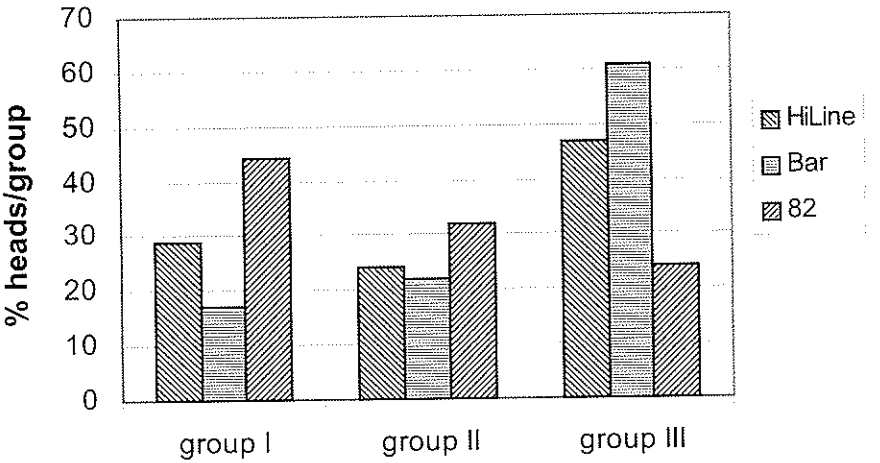

Figure 13.3. Levels of scab infection of transgenic wheat by *Fusarium culmorum*. Greenhouse experiment with HiLine transformed with *pinB* using the maize ubiquitin promoter. Bar is a transgenic line with glufosinate resistance but no *pin* gene. Group I is heads with 0–15% of the spikelets infected; Group II has 15–40% infected spikelets; and Group III has 40–100% infected spikelets.

membranes. The site-specific localization of these proteins to the starch granule membrane surface may be mediated by their hydrophobic tryptophan-rich domains. The high affinity of PINA and PINB for membrane lipids may be partly responsible for the antimicrobial properties of these two proteins.

It is well accepted that proteins that are structurally amphiphilic, hydrophobic, or capable of binding lipids may have inherent microbiocidal properties. Thionins, defensins, and non-specific lipid transfer proteins (nsLTPs) are the most common plant proteins reported to have antimicrobial properties (reviewed in Garcia-Olmedo *et al.*, 1995 and Broekaert *et al.*, 1997). All of these proteins can be characterized as small, basic, cysteine-rich proteins. Both PINA and PINB have the general structural characteristics of antimicrobial proteins in that they are small (~13 kDa), basic (pI >10), and cysteine rich, each having five disulphide bonds. Structurally, PINA and PINB are very similar to the nsLTP of wheat, a 9 kDa protein that shares significant structural similarity to the puroindolines. The additional sequence present in the PINA and PINB proteins consists of a unique tryptophan-rich domain. This tryptophan-rich region is quite hydrophobic, and is thought to be responsible for the lipid-binding properties of both PINA and PINB. These lipid-binding properties could be expected to disrupt lipid bilayer membranes of foreign organisms invading plant tissue. Binding to and permeabilization of membranes is believed to be the mechanism by which defensins cause cell death (Kagan *et al.*, 1990; Hill *et al.*, 1991). Thevissen *et al.* (1999) demonstrated that *Neurospora crassa* membranes were made permeable to the dye Sytox Green after exposure to several plant defensins. Therefore, puroindolines might cause similar disruption of membranes in invading pathogens. Both wheat nsLTPs and puroindolines have antimicrobial properties *in vitro* (Molina *et al.*, 1996; Dubreil *et al.*, 1998). However, the addition of a membrane-binding domain to the common structure shared between LTP and puroindolines (Marion *et al.*, 1994)

would suggest that the puroindoline proteins may be more effective in transgenic plant experiments in reducing pathogen growth. Transgenic tobacco plants with constitutive expression of the barley LTP2 protein (Molina and Garcia-Olmedo, 1997), or the barley alpha-thionin (Carmona *et al.*, 1993) showed much reduced bacterial pathogen infection on leaf tissue. Overexpression of endogenous thionin genes in *Arabadopsis* (Epple *et al.*, 1997) has also resulted in reduced wilt symptoms upon infection by *Fusarium oxysporum*. In addition, some of these genes have been demonstrated to be inducible by pathogen infection (Molina *et al.*, 1996). To date, transgenic rice and wheat with constitutive expression of either PINA or PINB in leaf tissue showed reduced disease symptoms in response to several fungal pathogens. There is good reason to propose that diseases caused by a wide range of pathogens of any crop plant might be controlled in this way.

References

BAKER, R.J. (1977). Inheritance of kernel hardness in spring wheat. *Crop Science* **17**, 960–962.

BALCONI, C. AND SHERWOOD, J.E. (2001). *In vitro* antifungal activity of the wheat puroindoline proteins. *Phytopathology* **91**, S5.

BARLOW, K.K., BUTTROSE, M.M., SIMMONDS, D.H. AND VESK, M. (1973). The nature of the starch–protein interface in wheat endosperm. *Cereal Chemistry* **50**, 443–454.

BEECHER, B., BETTGE, A., SMIDANSKY, E. AND GIROUX, M.J. (2002). Expression of wild-type pinB sequence in transgenic wheat complements a hard phenotype. *Theoretical and Applied Genetics* **105**, 870–877.

BLECHL, A.E. AND ANDERSON, O.D. (1996). Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. *Nature Biotechnology* **14**, 875–879.

BLOCHET, J.-E., CHEVALIER, C., FOREST, E. *ET AL.* (1993). Complete amino acid sequence of puroindoline, a new basic and cysteine-rich protein with a unique tryptophan-rich domain, isolated from wheat endosperm by Triton X-114 phase partitioning. *FEBS Letters* **329**, 336–340.

BORDIER, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. *Journal of Biological Chemistry* **256**, 1604–1607.

BROEKAERT, W.F., CAMMUE, B.P.A., DE BOLLE, M.F.C., THEVISSEN, K., DE SAMBLANX, G.W. AND OSBORN, R.W. (1997). Antimicrobial peptides from plants. *Critical Reviews in Plant Sciences* **16**, 297–323.

CAMPBELL, K.G., BERGMAN, C.J., GAULBERTO, D.G. *ET AL.* (1999). Quantitative trait loci associated with kernel traits in a soft × hard wheat cross. *Crop Science* **39**, 1184–1195.

CAMPBELL, K.G., FINNEY, P.L., BERGMAN, C.J. *ET AL.* (2001). Quantitative trait loci associated with milling and baking quality in a soft × hard wheat cross. *Crop Science* **41**, 1275–1285.

CARMONA, M.J., MOLINA, A., FERNANDEZ, J.A., LOPEZ-FANDO, J.J. AND GARCIA-OLMEDO, F. (1993). Expression of the alpha-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. *Plant Journal* **3**, 457–462.

CHEN, L.-F., BAAI, G.-H. AND DESJARDINS, A.E. (1999). Recent advances in wheat head scab research in China. Evaluation methods. http://www.nal.usda.gov/pgdic/WHS/eval.html.

DUBREIL, L.S., MELIANDE, H., CHIRON, J.P. *ET AL.* (1998). Effect of puroindolines on the breadmaking properties of wheat flour. *Cereal Chemistry* **75**, 222–229.

EPPLE, P., APEL, K. AND BOHLMANN, H. (1997). Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. *Plant Cell* **9**, 509–520.

GARCIA-OLMEDO, F., MOLINA, A., SEGURA, A. AND MORENO, M. (1995). The defensive role of nonspecific lipid transfer proteins in plants. *Trends in Microbiology* **3**, 72–74.

GARNIER, J., GIBRAT, J.F. AND ROBSON, B. (1996). GOR method for predicting protein secondary structure from amino acid sequence. *Methods in Enzymology* **266**, 540–553.

GATINEAU, E., TOMA, F., MONTENAY-GARESTIER, T., TAKECHI, M., FROMAGEOT, P. AND

MENEZ, A. (1987). Role of tyrosine and tryptophan residues in the structure–activity relationships of a cardiotoxin from *Naja nigricollis* venom. *Biochemistry* **26**, 8046–8055.

GAUTIER, M.-F., ALEMAN, M.-E., GUIRAO, A., MARION, D. AND JOUDIER, P. (1994). *Triticum aestivum* puroindolines, two basic cysteine-rich seed proteins: cDNA analysis and developmental gene expression. *Plant Molecular Biology* **25**, 43–57.

GAUTIER, M.-F., COSSON, P., GUIRAO, A., ALARY, R. AND JOUDRIER, P. (2000). Puroindoline genes are highly conserved in diploid ancestor wheats and related species but absent in tetraploid *Triticum* species. *Plant Science* **153**, 81–91.

GERHARDT, S.A., BALCONI, C. AND SHERWOOD, J.E. (2002). Control of fusarium scab with puroindoline-containing transgenic wheat. *Phytopathology* **92**, S28.

GIROUX, M.J. AND MORRIS, C.F. (1997). A glycine to serine change in puroindoline b is associated with wheat grain hardness and low levels of starch-surface friabilin. *Theoretical and Applied Genetics* **95**, 857–864.

GIROUX, M.J. AND MORRIS, C.F. (1998). Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6262–6266.

GIROUX, M.J., TALBERT, L., HABERNICHT, D.K., LANNING, S., HEMPHILL, A. AND MARTIN, J.M. (2000). Association of puroindoline sequence type and grain hardness in hard red spring wheat. *Crop Science* **40**, 370–374.

GREENBLATT, G.A., BETTGE, A.D. AND MORRIS, C.F. (1995). The relationship among endosperm texture, friabilin occurrence, and bound polar lipids on wheat starch. *Cereal Chemistry* **72**, 172–176.

GREENWELL, P. AND SCHOFIELD, J.D. (1986). A starch granule protein associated with endosperm softness in wheat. *Cereal Chemistry* **63**, 379–380.

HILL, C.P., YEE, J., SELSTED, M.E. AND EISENBERG, D. (1991). Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. *Science* **251**, 1481–1485.

JAMESON, B.A. AND WOLF, H. (1988). The antigenic index: a novel algorithm for predicting antigenic determinants. *CABIOS* **4**, 181–186.

JOLLY, C.J., RAHMAN, S., KORTT, V. AND HIGGINS, T.J.V. (1993). Characterization of the wheat Mr 15 000 grain-softness protein and analysis of the relationship between its accumulation in the whole seed and grain softness. *Theoretical and Applied Genetics* **86**, 589–597.

JOLLY, C.J., GLENN, G.M. AND RAHMAN, S. (1996). GSP-1 genes are linked to the grain hardness locus (*Ha*) on wheat chromosome 5D. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 2408–2413.

KAGAN, B.L., SELSTED, M.E., GANZ, T. AND LEHRER, R.I. (1990). Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 210–214.

KNOWLTON, K.F., ALLEN, M.S. AND ERICKSON, P.S. (1996). Lasalocoid and particle size of corn grain for dairy cows in early lactation. I. Effect of performance, serum metabolites, and nutrient digestibility. *Journal of Dairy Science* **79**, 557–564.

KRISHNAMURTHY, K. AND GIROUX, M.J. (2001). Expression of wheat puroindoline genes in transgenic rice enhances grain softness. *Nature Biotechnology* **19**, 1–5.

KRISHNAMURTHY, K., BALCONI, C., SHERWOOD, J.E. AND GIROUX, M. (2001). Increased tolerance to fungal diseases of rice plants transformed with puroindoline genes. *Molecular Plant–Microbe Interactions* **14**, 1255–1260.

LAW, C.N., YOUNG, C.F., BROWN, J.W.S., SNAPE, J.W. AND WORLAND, J.W. (1978). The study of grain protein control in wheat using whole chromosome substitution lines. In: *Seed protein improvement by nuclear techniques*, pp 483–502. Vienna, Austria: International Atomic Energy Agency.

LILLEMO, M. AND MORRIS, C.F. (2000). A leucine to proline mutation in puroindoline b is frequently present in hard wheats from Northern Europe. *Theoretical and Applied Genetics* **100**, 1100–1107.

LILLEMO, M. AND RINGLUND, K. (2002). Impact of puroindoline b alleles on the genetic variation for hardness in soft × hard wheat crosses. *Plant Breeding* **121**, 210–217.

MARION, D., GAUTIER, M.F., JOUDRIER, P. *ET AL.* (1994). Structure and function of wheat lipid binding proteins. In: *Wheat kernel proteins – molecular and functional aspects*. Ed. S. Martino al Cimino, pp 175–180. Universita degli studi della tuscia. 28–30 September, 1994. Viterbo, Italy.

MARTIN, J.M., FROHBERG, R.C., MORRIS, C.F., TALBERT, L.E. AND GIROUX, M.J. (2001). Milling and bread baking traits associated with puroindoline sequence type in hard red spring wheat. *Crop Science* **41**, 228–234.

MATTERN, P.J., MORRIS, R., SCHMIDT, J.W. AND JOHNSON, V.A. (1973). Location of genes for kernel properties in the wheat cultivar 'Cheyenne' using chromosome substitution lines. In: *Proceedings of the 4th International Wheat Genetics Symposium* (Columbia, MO, 1–6 August, 1973). Eds. E.R.Sears and L.M.S. Sears, pp 703–707.Columbia, USA: Agricultural Experiment Station, University of Missouri.

MOLINA, A. AND GARCIA-OLMEDO, F. (1997). Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. *Plant Journal* **12**, 669–675.

MOLINA, A., DIAZ, I., VASIL, I.K., CARBONERO, P. AND GARCIA-OLMEDO, F. (1996). Two cold-inducible genes encoding lipid transfer protein LTP4 from barley show differential responses to bacterial pathogens. *Molecular and General Genetics* **252**, 162–168.

MORRIS, C.F. (2002). Puroindolines: the molecular genetic basis of wheat grain hardness. *Plant Molecular Biology* **481**, 633–647.

MORRIS, C.F. AND ROSE, S.P. (1996). Wheat. In: *Cereal grain quality*. Eds, R.J. Henry and P.S. Kettlewell, pp 3–54. New York, USA: Chapman and Hall.

MORRIS, C.F., GREENBLATT, G.A., BETTGE, A.D. AND MALKAWI, H.I. (1994). Isolation and characterization of multiple forms of friabilin. *Journal of Cereal Science* **21**, 167–174.

MORRIS, C.F., LILLEMO, M., SIMEON, G.M., GIROUX, M.J., BABB, S. AND KIDWELL, K. (2001). Prevalence of puroindoline grain hardness genotypes among historically significant North American spring and winter wheats. *Crop Science* **41**, 218–228.

RAHMAN, S., JOLLY, C.J., SKERRITT, J.H. AND WALLOSHECK, A. (1994). Cloning of a wheat 15 kDa grain softness protein (GSP). GSP is a mixture of puroindoline-like polypeptides. *European Journal of Biochemistry* **223**, 917–925.

SCHIFFER, M., CHANG, C.H. AND STEVENS, F.J. (1992). The functions of tryptophan residues in membrane proteins. *Protein Engineering* **5**, 213–214.

SOURDILLE, P., PERRETANT, M.R., CHARMET, G. *ET AL.* (1996). Linkage between RFLP markers and genes affecting kernel hardness in wheat. *Theoretical and Applied Genetics* **93**, 580–586.

STACK, R.W. AND MCMULLEN, M.P. (1998). A visual scale to estimate severity of fusarium head blight in wheat. Publication No. PP1095. North Dakota State University Extension Service.

SYMES, K.J. (1965). The inheritance of grain hardness in wheat as measured by the particle size index. *Australian Journal of Agricultural Research* **16**, 113–123.

SYMES, K.J. (1969). Influence of a gene causing hardness on the milling and baking quality of two wheats. *Australian Journal of Agricultural Research* **20**, 971–979.

THEVISSEN, K., TERRAS, F.R.G. AND BROEKAERT, W.F. (1999). Permeabilization of fungal membranes by plant defensins inhibits fungal growth. *Applied and Environmental Microbiology* **65**, 5451–5458.

THORGEIRSSON, T.E., RUSSEL, C.J., KING, D.S. AND SHIN, Y.K. (1996). Direct determination of the membrane affinities of individual amino acids. *Biochemistry* **35**, 1803–1809.

TRANQUILLI, G., LIJAVETZKY, D., MUZZI, G. AND DUBCOVSKY, J. (1999). Genetic and physical characterization of grain texture-related loci in diploid wheat. *Molecular and General Genetics* **262**, 846–850.

TURNBULL, K.-M., GABORIT, T., MARION, D. AND RAHMAN, S. (2000). Variation in puroindoline polypeptides in Australian wheat cultivars in relation to grain hardness. *Australian Journal of Plant Physiology* **27**, 53–158.

WONDRA, K.J., HANCOCK, J.D., BEHNKE, K.C., HINES, R.H. AND STARK, C.R. (1995). Effects of particle size and pelleting on growth performance, nutrient digestibility, and stomach morphology in finishing pigs. *Journal of Animal Science* **73**, 757–763.