ORIGINAL ARTICLE

The promoter of the wheat puroindoline-a gene (*PinA*) exhibits a more complex pattern of activity than that of the *PinB* gene and is induced by wounding and pathogen attack in rice

Alexandre Evrard · Donaldo Meynard · Emmanuel Guiderdoni · Philippe Joudrier · Marie-Françoise Gautier

Abstract Puroindolines form the molecular basis of wheat grain hardness. However, little is known about puroindoline gene regulation. We previously reported that the *Triticum aestivum* puroindoline-b gene (*PinB*) promoter directs β-glucuronidase gene (*uidA*) seed-specific expression in transgenic rice. In this study, we isolated a puroindoline-a gene (*PinA*), analyzed *PinA* promoter activity by 5′ deletions and compared *PinA* and *PinB* promoters in transgenic rice. Seeds of PinA-1214 and PinB-1063 transgenic plants strongly expressed *uidA* in endosperm, in the aleurone layer and in epidermis cells in a developmentally regulated manner. The GUS activity was also observed in PinA-1214 embryos. Whereas the *PinB* promoter is seed specific, the *PinA* promoter also directed, but to a lower level, *uidA* expression in roots of seedlings and in the vascular tissues of palea and pollen grains of dehiscent anthers during flower development. In addition, the *PinA* promoter was induced by wounding and by *Magnaporthe grisea*. By deletion analysis, we showed that the "390-bp" *PinA* promoter drives the same expression pattern as the "1214-bp" promoter. Moreover, the "214-bp" *PinA* promoter drives *uidA*

expression solely in pollen grains of dehiscent anthers. The presence of putative *cis*-regulatory elements that may be related to *PinA* expression is discussed from an evolutionary point of view. By electrophoretic mobility shift assay, we showed that putative *cis*-elements (WUN-box, TCA motifs and *as*-1-like binding sites) whose presence in the *PinA* promoter may be related to wounding and/or the pathogen response form complexes with nuclear extracts isolated from wounded wheat leaves.

Keywords Plant defense · Promoter analysis · Puroindoline-gene regulation · Transgenic rice · *uidA*-gene reporter · Wheat

Abbreviations

DAF	Days after flowering
DAG	Days after germination
EMSA	Electrophoretic mobility shift assay
GUS	β-Glucuronidase
hfw	Hours following wounding
hpi	Hours post-inoculation
MATAB	Mixed alkyl trimethyl ammonium bromide
PinA	*Triticum aestivum* puroindoline-a gene
PIN-a	Puroindoline-a
PinB	*T. aestivum* puroindoline-b gene
PIN-b	Puroindoline-b
PCR	Polymerase chain reaction
UidA	β-Glucuronidase gene
UTR	Untranslated region

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Introduction

Puroindolines are small basic proteins (13 kDa) that were isolated from *Triticum aestivum* flour as a

component of an enriched lipid and membrane protein fraction (Blochet et al. 1993). Two proteins, puroindoline-a (PIN-a) and puroindoline-b (PIN-b) showing 60% of identity are characterized by a cystein-rich backbone and a tryptophan-rich domain (Blochet et al. 1993; Gautier et al. 1994). Since their finding, puroindolines have created considerable interest because of their attractive properties. First, due to their capacity to bind lipids, puroindolines display exceptional tensioactive and foaming properties (Clark et al. 1994; Dubreil et al. 1998) making them interesting natural surfactants for the agro industry. Second, puroindolines form the molecular basis of wheat grain hardness (for review see Morris 2002) which is an important criterion for commercial transactions and determines wheat end-use quality. The report of a major QTL of wheat grain friability associated with the *PinA* locus (Sourdille et al. 1996), of SNPs in *PinB* sequence or *PinA* deletion related to friability (Giroux and Morris 1997, 1998; Tranquilli et al. 2002) and the modification of transgenic rice (Krishnamurthy and Giroux 2001) and wheat (Beecher et al. 2002; Hogg et al. 2005) grain texture overexpressing puroindolines, clearly demonstrated the role of puroindolines in this character. Third, puroindolines display antimicrobial and antibacterial activities in vitro (Dubreil et al. 1998; Capparelli et al. 2005) and increase resistance to plant pathogens in transgenic rice (Krishnamurthy et al. 2001) and apple (Faize et al. 2004) expressing puroindolines. Despite this fact, the cellular functions of puroindolines are still unclear. Two main functions have been proposed in wheat in which puroindolines form the molecular basis of grain hardness and their antifungal/antibacterial properties may contribute to plant defense.

Until now, most research on puroindoline genes was conducted using a genetic approach. *PinA* and *PinB* genes are located on the short arm of chromosome 5D (Sourdille et al. 1996) at the *Ha* locus that controls grain hardness (Mattern et al. 1973; Law et al. 1978) and also contains the *Gsp-1* gene coding for the grain softness protein (Rahman et al. 1994). These three genes were shown to be closely linked in the diploid wheat species *T. monococcum* (Tranquilli et al. 1999) and *Aegilops tauschii* (Turnbull et al. 2003) and this locus was sequenced in *T. monococcum* (Chantret et al. 2004), *T. aestivum* (Chantret et al. 2005) and barley (Caldwell et al. 2004). *PinA* and *PinB* genes are present in all the diploid wheat species tested so far but absent in the tetrapoid species *T. turgidum* ssp. *durum* as well as in the A and B genomes of the hexaploid bread wheat (Gautier et al. 2000; Lillemo et al. 2002). *PinA* and *PinB* loss was due to independent deletions

of several kilobases in A and B genomes (Chantret et al. 2005). Ortholog genes are also present in barley, rye and oats (Gautier et al. 2000). However in barley, *Gsp-1*, *PinA* and *PinB* orthologous genes were not found in the same order as in wheat (Caldwell et al. 2004; Chantret et al. 2005).

In contrast, few studies have focused on puroindoline gene regulation, although Digeon et al. (1999) showed that the *PinB* promoter is seed specific and drives *uidA* expression in aleurone, epidermis cells and starchy endosperm of rice seeds in a developmentally regulated manner. Up to now promoter regions have been isolated for *PinA* and *PinB* genes (Digeon et al. 1999; Lillemo et al. 2002) and in silico analysis of *PinA* and *PinB* gene promoters carried out on ancestor wheats (Lillemo et al. 2002), but there are no data on *PinA* promoter activity. In this study we isolated the wheat *PinA* gene and investigated its promoter activity in transgenic rice using the *uidA* reporter gene. Analysis of 5′ deletions of the *PinA* promoter led to the identification of regions involved in tissue specificity. Moreover, we analyzed in parallel and under the same conditions the expression pattern directed by *PinA* and *PinB* promoters.

Materials and methods

Molecular techniques and sequence analysis

The *PinA* gene was isolated by screening a genomic library of *T. aestivum* L. (cv. Chinese Spring) leaves by colony hybridization using a ^{32}P-labeled PCR product of the pTa31 cDNA encoding PIN-a (Gautier et al. 1994) as probe. The genomic library constructed into the λFixII vector (Stratagene) was a gift of Dr. C. Hartmann (Université Paris VII, France). Phage DNA fragments hybridizing to the *PinA* probe were directly sequenced using an automated DNA sequencer (373 DNA sequencer stretch, Applied Biosystems). From the sequencing results, a fragment of 1,664 bp of the *PinA* gene was amplified by PCR using phage DNA and ppa1-a (5′-GTTTGAATTCTGATCTGCATGA CTGTGTGC-3′) and AM4ep (5′-CGCGGATCCA CATCACCAGTAATAGC-3′) primers. For promoter analysis, primers ppa1-a (5′-GTTTGAATTCTG ATCTGCATGACTGTGTGC-3′), ppa2 (5′-TCTAG AGAATTCACGAAAAAGCAGTGGCTAGAAA GA-3′), ppa3 (5′-ACAAGAATTCATGGTTTTA TTTTGAGAAAAGGTC-3′) or ppa4 (5′-TTTTG AATTCTTTCAAAGTAACTTTGATTGGTATCC-3′) associated with IpuroA (5′-GTCGACCATGTTGT-CAGTGTGTTTTGG-3′) were used to PCR amplify

phage DNA, generating four fragments of 1,214, 390, 214 and 136 bp of the *PinA* 5′ UTR. Primer ppb (5′-CCAAGAATTCAACATCTTATCGCAACATCC-3′) and IpuroB (5′-GTCGACCATGTTTTCAATGTTGTTTGGTGGTCC-3′) were used to PCR amplify *PinB* 5′ UTR as previously described (Digeon et al. 1999). All these PCR fragments were subcloned in the pGEM-Teasy vector (Promega) to obtain the pGppa1-a, pGppa2, pGppa3 and pGppa4 plasmids for *PinA* constructs and pGppb plasmid for *PinB*. After verification of sequences by sequencing, DNA fragments were digested and inserted as an *Eco*RI/*Sal*I fragment into the pCAMBIA 1381Xb vector (R. Jefferson, CAMBIA, Canberra, Australia) upstream of the bacterial *uidA* gene (encoding the GUS protein) resulting in the pCppa1-a, pCppa2, pCppa3, pCppa4 and pCppb plasmids.

Comparison of *PinA* and *PinB* promoter sequences was performed using the ClustalW program (http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl). We searched for putative regulatory *cis*-elements in the "390-bp" promoter region of *PinA* and *PinB* genes using the TFSEARCH (http://www.genome.ad.jp/SIT) and PlantCARE (Lescot et al. 2002) databases, as well as a search of the literature.

Rice transformation and selection of transgenic plants

pCppa1-a, pCppa2, pCppa3, pCppa4, pCppb and pCAMBIA 1381Xb (pC-Xb) plasmids were introduced into *Agrobacterium tumefaciens* strain EHA105 to transform *japonica* rice (*Oryza sativa* L.) cv. Zhongzuo321 (seeds were kindly supplied by Dr ZL Chen, Beijing University, Beijing, China) through co-culture of seed-embryo callus (Sallaud et al. 2003). The corresponding regenerated hygromycin-resistant plants were named PinA-1214, PinA-390, PinA-214, PinA-136, PinB-1063 and pC-Xb, respectively. Depending on the constructs, the frequency of regeneration ranged from 34.7 to 59.6%. For each construct, 86–169 plants were regenerated and 60 T0 plants were analyzed by Southern blot to determine the T-DNA copy number. Genomic DNA was extracted from 200 mg of fresh 4-week-old rice leaves using 800 μl of MATAB buffer (100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% [w/v] MATAB, 1% [w/v] PEG 6000, 0.5% [w/v] Na_2SO_2) prewarmed at 72°C. The mix was vortexed for 10 s, incubated for 45 min at 72°C, extracted with chloroform, treated with RNase A, extracted with chloroform and then DNA was ethanol precipitated. Five micrograms of genomic DNA were digested overnight with *Eco*RI, fractionated through a 0.8% agarose gel and alkali-transferred onto

Hybond *N*+ nylon membrane (Amersham Biosciences). Membranes were hybridized with an α-^{32}P-labeled ppa3/IpuroA PCR fragment as probe for plants carrying *PinA* promoter constructs and an α-^{32}P-labeled ppb/IpuroB PCR fragment as probe for plants carrying the *PinB* promoter construct. Autoradiography of membranes was performed using X-Ray film at −80°C. Eight to 15 plants harbouring a single copy of the transgene were allowed to grow in the greenhouse to generate T1 and T2 seeds for further analysis.

GUS assays

Histochemical and fluorometric GUS assays were performed with basic techniques as described by Jefferson et al. (1987). For histological analysis, stained samples were incubated in a fixative solution (200 mM NaH_2PO_4, pH 7, 1% [w/v] acrolein, 2% [w/v] glutaraldehyde, 1% [w/v] cafein) for 30 min under vacuum and left for 48 h at 4°C. Samples were then cleared through a graded ethanol series or prepared for section. Sections and fluorometric assays were done as described (Guiderdoni et al. 2002). The GUS assays were performed on T1 plants except for seed tissue assays, which were carried out on T2 progenies.

Wounding and infection treatments

Rice

To determine whether *PinA* promoter activity is altered in response to mechanical wounding, leaf tissues of rice seedlings exhibiting 6–7 expanded leaves were wounded using a multineedle system allowing to dispense 60 punctures on a 3 cm^2 area. Rank 2 leaves (considered as old) and rank 5 leaves (young) were wounded using the needle system and the unwounded rank 4 leaf was used as systemic sample. Tissues were collected 0, 2, 4 and 8 h following wounding (hfw), immediately frozen in liquid nitrogen and kept at −80°C. For histological analysis of GUS staining, a razor blade was used to perform the mechanical injuries (leaf) or sampling (stem and flower peduncle). For plant infection, transgenic plants were inoculated with *M. grisea* (FR13) by spraying 30 ml of an aqueous suspension of spores (10^5 spores ml^{-1}) supplemented with 0.5% (w/v) gelatin on seedlings at the 4–5 leaf stage. Controls were sprayed with a solution of 0.5% (w/v) gelatin only. For fluorometric GUS analysis, plant samples were harvested at 36 h post-inoculation (hpi) and frozen in liquid nitrogen. Histochemical GUS assays were performed at 72 hpi to visualize response to infection.

Wheat

Triticum aestivum L. cv. Chinese Spring (seeds were kindly supplied by Dr. T.J. Close, University of California, Davis, USA) seedlings were grown on soil for 4 weeks in a growth chamber under a 10-h day and 14-h night regime at 21°C and 100 μmol m^{-2} s^{-1} photons. Wounding of 4-week-old wheat leaves and collection of samples were performed as described above for rice.

Electrophoretic mobility shift assays

WunpinAF 5′-TACCTAGAAAAATACAATATCT AATTTCCTCTTGAT-3′ and WunpinAR 5′-ATC AAGAGGAAATTAGATATTGTATTTTTCTAG GTA-3′ oligonucleotides were annealed at a concentration of 50 pmol μl^{-1} in water by heating at 95°C for 5 min and left at room temperature for 1 h to form the *PinA*-WUN probe. WBpinAF 5′-AAAAGCAGTG GCTAGAAAGATGACGATATATA-3′ and WBpinAR 5′-TATATATCGTCATCTTTCTAGCCAC TGCTTTT-3′ were annealed in the same conditions to form the *PinA*-AS1 probe. Nuclear protein extracts from wounded wheat leaves (at 0, 2, 4 and 8 hfw) were prepared as previously described (Desveaux et al. 2004). Twenty-five pmol of annealed oligonucleotides were end-labeled by 2 units of T4 polynucleotide kinase, 9.2×10^6 Bq [γ-^{32}P]-dATP (Amersham Biosciences). Unincorporated [γ-^{32}P]-dATP was removed with a Sephadex™ G-50 column (Amersham Biosciences). To assess DNA-binding properties, 10 μg of nuclear protein extracts were mixed with 30,000 cpm of radio-labeled probe, with or without unlabeled probe (80 time molar excess), in EMSA binding buffer supplemented with 1 mM DTT and 500 ng of poly (dI-dC; Roche) in a final volume of 25 μl. The same conditions without addition of the nuclear protein extracts were used as a control reaction. After being incubated for 20 min at room temperature, 5 μl of loading dye were added to each reaction. Samples were run on a nondenaturing 5.4% polyacrylamide gel (45.9 ml H$_2$O, 6 ml 5× TBE, 8.1 ml of 40% acrylamide [29:1 acrylamide/bis-acrylamide], Fisher Scientific). After running for 2 h at 200 V, gels were exposed to X-ray autoradiography films (Kodak) at −80°C with intensifying screens.

Results

Cloning and structural features of the *PinA* gene

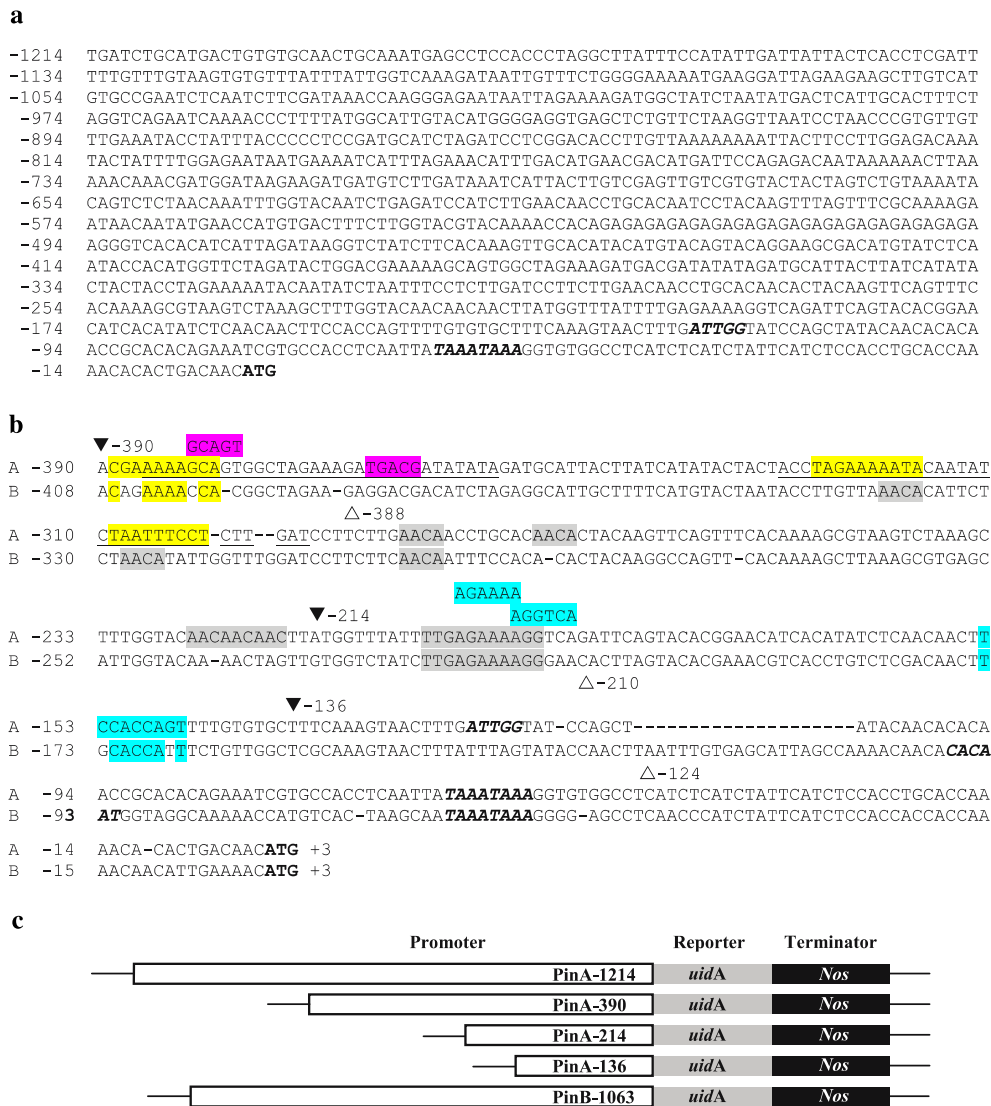Using the pTa31 insert encoding PIN-a (Gautier et al. 1994) as a probe to screen a wheat (*T. aestivum*, cv Chinese Spring) genomic library, eight clones were isolated, one of which was subsequently used to determine the sequence of the *PinA* gene. As already known, the *PinA* gene is intronless; the sequence spanning 1,214 bp upstream from the translation start codon is presented in Fig. 1a. The 875 bp upstream from the ATG are identical to those of the *PinA* promoter isolated from *T. aestivum* cv. Penawawa (Lillemo et al. 2002). Further analysis of the *PinA* promoter sequence revealed several putative *cis*-regulatory elements that are listed in Table 1. The 1,070 bp of the 5′ UTR of *PinA* and *PinB* genes displayed 48% identity (data not shown), whereas the 400 bp upstream from the start codon appeared to be more conserved with 66% identity (Fig. 1b). In this region, the major difference lies in a "20-bp" deletion in the *PinA* promoter at position −106 compared to the *PinB* promoter. A microsatellite sequence consisting of the dinucleotide GA motif repeated 18 times was only found in the *PinA* promoter sequence at position −495. Putative TATA boxes were found to be well conserved but not the putative CAAT box.

Production and selection of transgenic rice

To analyze the tissue specificity and the developmental regulation of the *PinA* gene, four fragments of the promoter region (1,214, 390, 214 and 136 bp) were amplified by PCR and cloned into the pCAMBIA 1381Xb vector resulting in transcriptional fusions with the *uidA* gene (Fig. 1c). Although the *PinB* gene has already been studied (Digeon et al. 1999), 1,063 bp of its promoter were also cloned into the pCAMBIA vector in order to compare *PinA* and *PinB* promoter activity in the same rice cultivar and in identical experimental conditions.

These constructs were introduced into the rice cultivar Zhongzuo 321 by cocultivation with *A. tumefaciens*. For each construct, 60 hygromycin-resistant lines were regenerated and further investigated by Southern blot analysis to determine the transgene copy number. Data from Southern blot analysis indicated that, depending on the construct, 8–15 transformed rice plants derived from independently transformed cell lines harboured a single copy of the transgene. These lines were propagated and grown in the greenhouse until seed maturity.

As puroindoline genes are known to be expressed in bread wheat seeds, we first analyzed transgenic rice seeds for GUS activity by histochemical assay. The percentages of transgenic rice plants expressing the *uidA* gene in T1 seeds were 73% (11/15), 67% (8/12), 0% (0/15) and 0% (0/11) for PinA-1214, PinA-390, PinA-214 and PinA-136, respectively. Sixty-three

a

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-1214 TGATCTGCATGACTGTGTGCAACTGCAAATGAGCCTCCACCCTAGGCTTATTTCCATATTGATTATTACTCACCTCGATT
-1134 TTTGTTTGTAAGTGTGTTTATTTATTGGTCAAAGATAATTGTTTCTGGGGAAAAATGAAGGATTAGAAGAAGCTTGTCAT
-1054 GTGCCGAATCTCAATCTTCGATAAACCAAGGGAGAATAATTAGAAAAGATGGCTATCTAATATGACTCATTGCACTTTCT
 -974 AGGTCAGAATCAAAACCCTTTTATGGCATTGTACATGGGGAGGTGAGCTCTGTTCTAAGGTTAATCCTAACCCGTGTTGT
 -894 TTGAAATACCTATTTACCCCCTCCGATGCATCTAGATCCTCGGACACCTTGTTAAAAAAAATTACTTCCTTGGAGACAAA
 -814 TACTATTTTGGAGAATAATGAAAATCATTTAGAAACATTTGACATGAACGACATGATTCCAGAGACAATAAAAAACTTAA
 -734 AAACAAACGATGGATAAGAAGATGATGTCTTGATAAATCATTACTTGTCGAGTTGTCGTGTACTACTAGTCTGTAAAATA
 -654 CAGTCTCTAACAAATTTGGTACAATCTGAGATCCATCTTGAACAACCTGCACAATCCTACAAGTTTAGTTTCGCAAAAGA
 -574 ATAACAATATGAACCATGTGACTTTCTTGGTACGTACAAAACCACAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
 -494 AGGGTCACACATCATTAGATAAGGTCTATCTTCACAAAGTTGCACATACATGTACAGTACAGGAAGCGACATGTATCTCA
 -414 ATACCACATGGTTCTAGATACTGGACGAAAAAGCAGTGGCTAGAAAGATGACGATATATAGATGCATTACTTATCATATA
 -334 CTACTACCTAGAAAAATACAATATCTAATTTCCTCTTGATCCTTCTTGAACAACCTGCACAACACTACAAGTTCAGTTTC
 -254 ACAAAAGCGTAAGTCTAAAGCTTTGGTACAACAACAACTTATGGTTTATTTTGAGAAAAGGTCAGATTCAGTACACGGAA
 -174 CATCACATATCTCAACAACTTCCACCAGTTTTGTGTGCTTTCAAAGTAACTTTG***ATTGG***TATCCAGCTATACAACACACA
  -94 ACCGCACACAGAAATCGTGCCACCTCAATTA***TAAATAAA***GGTGTGGCCTCATCTCATCTATTCATCTCCACCTGCACCAA
  -14 AACACACTGACAAC**ATG**
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b

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         ▼-390   GCAGT
A -390 A CGAAAAAGCA GTGGCTAGAAAGA TGACG ATATATAGATGCATTACTTATCATATACTACT ACC TAGAAAAATA CAATAT
B -408 A C AG AAAA CCA -CGGCTAGAA-GAGGACGGACATCTAGAGGCATTGCTTTTCATGTACTAATACCTTGTT AAACA CATTCT
                                 △-388
A -310   TAATTTCCT -CTT--GAT CCTTCTTG AACA ACCTGCAC AACA CTACAAGTTCAGTTTCACAAAAGCGTAAGTCTAAAGC
B -330 CTA ACA TATTGGTTTGGATCCTTCTTC AACA ATTTCCACA-CACTACAAGGCCAGTT-CACAAAAGCTTAAAGCGTGAGC
                                AGAAAA
                                AGGTCA
A -233 TTTGGTAC AACAACAAC TTATGGTTTATTTTGAGAAAGG T CAGATTCAGTACACGGAACATCACATATCTCAACAACT T
B -252 ATTGGTACAA-AACTAGTTGTGGTCTATC TTGAGAAAAGG GAACACTTAGTACACGAAACGTCACCTGTCTCGACAACT T
                                               △-210
         ▼-136
A -153 CCACCAGT TTTGTGTGCTTTCAAAGTAACTTTG***ATTGG***TAT-CCAGCT-------------------ATACAACACACA
B -173 G CACCATT TCTGTTGGCTCGCAAAGTAACTTTATTTAGTATACCAACTTAATTTGTGAGCATTAGCCAAAACAACA***CACA***
                                                        △-124
A  -94 ACCGCACACAGAAATCGTGCCACCTCAATTA***TAAATAAA***GGTGTGGCCTCATCTCATCTATTCATCTCCACCTGCACCAA
B  -93 ***AT***GGTAGGCAAAAACCATGTCAC-TAAGCAA***TAAATAAA***GGGG-AGCCTCAACCCATCTATTCATCTCCACCACCACCAA

A  -14 AACA-CACTGACAAC**ATG** +3
B  -15 AACAACATTGAAAAC**ATG** +3
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c

	Promoter	Reporter	Terminator
	PinA-1214	*uid*A	*Nos*
	PinA-390	*uid*A	*Nos*
	PinA-214	*uid*A	*Nos*
	PinA-136	*uid*A	*Nos*
	PinB-1063	*uid*A	*Nos*

Fig. 1 Sequence, structural features of the *PinA* gene promoter and comparison of the 400 bp upstream from the ATG of *PinA* and *PinB* promoters. **a** Sequence of the "1214-bp" *PinA* promoter (accession number CS131558). The start codon and putative TATA and CAAT boxes are in *bold and italics*. **b** Comparison of *PinA* (A) and *PinB* (B) promoter sequences. Position of deletions is marked with *down filled triangle* for *PinA* and *open up triangle* for *PinB*. Putative *cis*-elements regulating *PinA* promoter expression in seeds and flowers are highlighted in *gray and blue*, respectively. Putative *cis*-elements regulating *PinA* promoter expression in response to wounding or involved in plant defense are highlighted in *yellow and purple*. Sequences of *PinA*-WUN and *PinA*-AS1 probes used for the electrophoretic mobility shift assay (EMSA) are *underlined*. Details of specific *cis*-element features are listed in Table 1. **c** Schematic representation (not to scale) of the *Pin::uidA* constructs. The *uidA* gene is driven by different lengths of the *PinA* (1,214, 390, 214, 136 bp) or *PinB* (1,063 bp) promoter and *Nos* terminator

percent (5/8) and 0% (0/10) of PinB-1063 and pC-Xb plants, respectively, exhibited GUS activity in seeds.

PinA promoter activity is temporally and spatially regulated in transgenic rice

Comparison of PinA and PinB promoter activity in transgenic rice seeds

PinA-1214 and PinA-390 plants exhibited GUS activity in developing seed tissues subjected to histochemical assay whereas no GUS activity was detected in seeds of PinA-214 and PinA-136 (Fig. 2a) or control pC-Xb (Fig. 2c) plants. Figure 2a, c illustrates observations of seed sections at 20 days after flowering (DAF) that agree with observations performed at 8, 15, 30 and 40 DAF (data not shown). These results indicate that the region spanning 214 bp upstream from the ATG of the *PinA* gene is not sufficient to direct *uidA* expression in seeds. As illustrated for PinA-1214 developing seeds, GUS staining was observed as early as at 8 DAF at the frontier zone between the endosperm and the

Table 1 Comparison of putative regulatory *cis*-elements in the *PinA* and *PinB* promoters (390 bp)

Motif	Sequence *PinA*/*PinB*	Position *PinA*/*PinB*
TATA	TAAATAAA/TAAATAAA	−56/−56
CAAT	ATTGG/CACAAT	−116/−92
P-box[a]	TTGAGAAAAGG	−194/−214
AACA[b]	AACA	−283/−271
		−219/−173
		−158/−300
		−325/−337
Flower related[c]	AGGTCA/AGGGAA	−191/−211
Flower related[d]	TCCACCAGT/TGCACCATT	−146/−166
	AGAAAA	−196/−216
*as*1-like[e]	GCAGT(n)₁₁TGACG	−362
TCA[f]	CGAAAAGCA/	−380/−398
	CAGAAAACCA	
	TAGAAAAATA	−317
WUN[g]	TAATTTCCT	−300

[a] Motto et al. (1989)

[b] Morton et al. (1995), Wu et al. (1998, 2000)

[c] Present in the maize *ZM13* gene (Hamilton et al. 1998)

[d] Present in the tomato *lat52* gene (Eyal et al. 1995)

[e] Rouster et al. (1997)

[f] Goldsbrough et al. (1993) and Pastuglia et al. (1997)

[g] Present in the potato *STH-2* and colza *SFR2* promoter (Matton et al. 1993; Pastuglia et al. 1997)

epithelium of the scutellum and tended to spread throughout the endosperm at more advanced development stages until 30 DAF (Fig. 2b). PinA-1214 and PinA-390 seeds displayed similar GUS activity patterns. Observation of 3 μm longitudinal sections of PinA-1214 seeds by light microscopy revealed that GUS staining (appearing pink in inverted light) was more intense in aleurone, testa and epidermal cell layers than in the endosperm (Fig. 2d, e). Intense GUS activity was also restricted to the frontier between the endosperm and the epithelium of the scutellum in the outermost cells (Fig. 2f).

Comparison of GUS staining in PinA-1214 (Fig. 2h, top) and PinB-1063 (Fig. 2h, bottom) seeds at 20 DAF revealed two main differences. The first difference was that GUS activity was more restricted to the periphery of the endosperm and inside aleurone cells in PinA-1214 seeds (Fig. 2i, top) than in PinB-1063 seeds in which more intense GUS staining was observed in the axial zone of the endosperm (Fig. 2i, bottom). The second difference was in GUS staining of embryo tissues, a close examination of which showed no GUS activity in PinB-1063 seed embryos (Fig. 2j, left) whereas GUS activity was detected in PinA-1214 seed embryos (Fig. 2j, right). Hence, in PinA-1214 embryos, GUS staining appeared dominant in the integument, in the external tissues of the coleoptile and leaf primordia

and in the tissues surrounding the coleorhiza (Fig. 2j, right). Observation by light microscopy of an isolated PinA-1214 embryo showed weak GUS staining in the leaf primordia (Fig. 2g) and in the root (not shown here).

Quantitative analysis of GUS activity in PinA-1214, PinA-390, PinA-214 and PinA-136 seeds confirmed the results of the histochemical assays. In PinA-1214 and PinA-390 seeds, GUS specific activity was maximum around 15 DAF (Fig. 2k). No GUS activity was measured at any developmental stage in PinA-214 and PinA-136 seeds or in control pC-Xb seeds (data not shown).

PinA promoter drives uidA expression in seedlings, roots, leaves and flowers of transgenic rice

We previously reported that *PinA* and *PinB* mRNA accumulation is restricted to seed tissues in bread wheat (Gautier et al. 1994). This result was further confirmed for the *PinB* gene promoter which directs seed-specific *uidA* expression in transgenic rice (Digeon et al. 1999). However, because of unexpected detection of GUS activity at the section of incised leaf samples of *PinA* transgenic plants, we carried out a complete gene expression analysis of *PinA* and *PinB* promoters and *PinA* promoter deletions in different organs and at different stages of rice development. For each construct, this analysis was performed during T1 seed germination and GUS staining was only detected in PinA-1214 and PinA-390 seedlings that displayed a similar GUS activity pattern. Representative results for PinA-1214 seedlings are presented in Fig. 3. Three days after germination (DAG) and from the early stages of coleoptile and seminal root elongation, intense GUS staining was restricted to the embryo and was strongly reduced in the endosperm (Fig. 3a and details in Fig. 3b) in contrast to what was observed in mature seeds (Fig. 2b). On the other hand, no GUS staining was observed in emerging organs. At 6 DAG, GUS activity was restricted to the embryo (Fig. 3c) and the vascular tissues at the proximal region of crown roots (Fig. 3d). At 15 DAG, the *PinA* promoter expression pattern changed and GUS staining was concentrated at the base of coleoptile mainly in vascular tissues (Fig. 3e), in the upper part of the grain (Fig. 3f), in primary roots (Fig. 3g) and secondary roots (Fig. 3h). Cross sections of primary roots revealed intense GUS staining in vascular tissues (Fig. 3m) and at the branching of secondary roots (Fig. 3n). No GUS staining was observed within the root tip and elongation zone. The GUS staining in roots of PinA-1214 and PinA-390 plants was detected throughout the life cycle
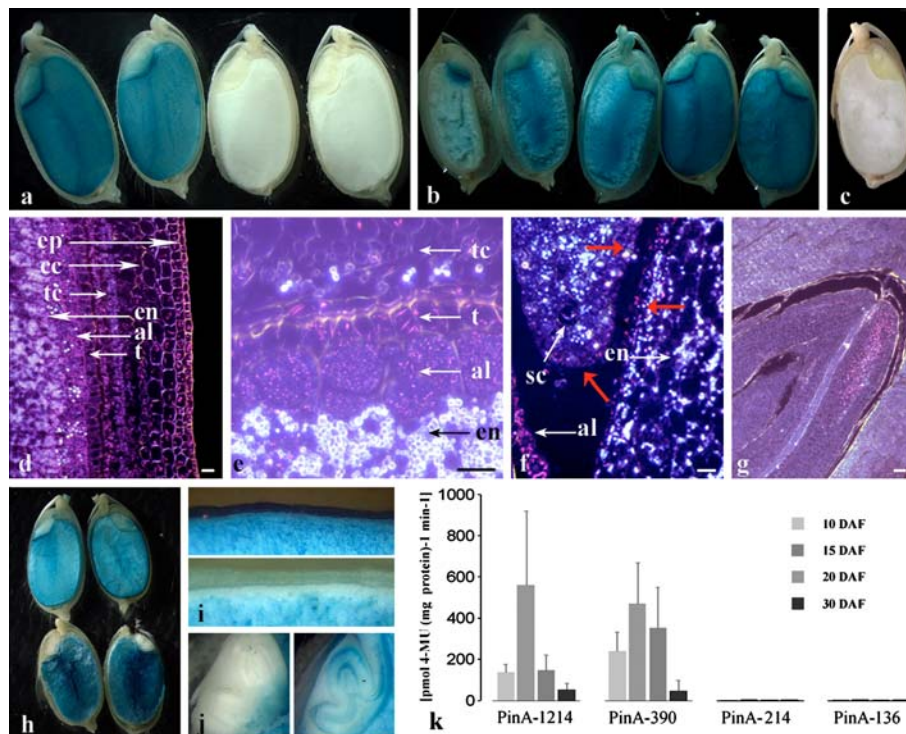
Fig. 2 Gene expression of *uidA* under the control of wheat *PinA* and *PinB* promoters in transgenic rice seeds. The results shown are representative of those observed in five independent transgenic lines developed for each gene construct during the different stages of seed maturation that were tested. **a–c** Histochemical localization of GUS activity in a longitudinal section of transgenic rice seeds. **a** Twenty DAF seeds from PinA-1214, PinA-390, PinA-214 and PinA-136 T2 plants (from left to right, respectively). **b** PinA-1214 seeds at 8, 10, 15, 20 and 30 DAF (from left to right, respectively). **c** Twenty DAF seeds from pC-Xb control plant. **d–g** Histological analysis on 3 µm sections of 20 DAF PinA-1214 seeds. **d** Ventral side (*al* aleurone cells, *cc* cross cell, *en* endosperm, *ep* epidermis, *t* testa, *tc* tube cell). **e** Detail of aleurone cells. **f** Frontier between the endosperm and epithelium of the scutellum (*sc* scutellum). **g** Isolated embryo. **h–j** Comparison of GUS activity in PinA-1214 and PinB-1063 seeds. **h** Longitudinal section of PinA-1214 (*top*) and PinB-1063 (*bottom*) seeds. **i** Closer view of endosperm, aleurone cells and epidermis of PinA-1214 (*top*) and PinB-1063 (*bottom*). **j** Closer view of PinA-1214 (*right*) and PinB-1063 (*left*) embryos. **k** Fluorometric analysis of GUS specific activities in protein extracts of PinA-1214, PinA-390, PinA-214 and PinA-136 T2 seeds during development. Each bar is representative of the average GUS activity calculated from a pool of five seeds from five different plant lines. *Bars*, 25 µm (**d**–**f**), 160 µm (**g**)

of the plant but decreased strongly after flowering (data not shown). No GUS staining was observed in primary (Fig. 3i) and secondary (Fig. 3j) roots of pC-Xb control plants. Plants carrying PinA-1214 and PinA-390 constructs displayed a similar expression pattern whereas no GUS activity was detected in PinA-214, PinA-136, PinB-1063 and pC-Xb seedlings (data not shown).

In leaves of PinA-1214 and PinA-390 transgenic rice, GUS staining was restricted to the collar region between sheath and blade as illustrated for PinA-1214 leaves at 15 DAG (Fig. 3k). In leaves of 3-month-old plants, GUS staining was restricted to the ligule scar region (Fig. 3l). Once again, no GUS staining was detected in PinA-214, PinA-136, PinB-1063 and pC-Xb leaves (data not shown).

The *uidA* expression directed by the *PinA* promoter appears to be both temporally and spatially regulated during rice flower development, as illustrated in Fig. 4. In PinA-1214 and PinA-390 developing flowers, no or very weak GUS staining was detected at early stages of development (not shown here). GUS staining, which was first localized in vascular tissues of the top half of the palea, gradually expanded to its lower half until the fertilization stage (Fig. 4a). GUS activity was detected in anthers only at the mature stage (Fig. 4b). No GUS staining was observed in anthers at earlier stages or after pollen release. A closer examination of anthers from PinA-1214 (Fig. 4c) and PinA-390 (Fig. 4d) flowers showed that the blue crystal precipitates were localized inside the pollen grains and at the dehiscence slit. In flowers of PinA-214 plants no GUS activity was detected in the palea irrespective of the development stage whereas GUS staining was restricted to anthers, in contrast to observations in PinA-1214 and PinA-390 flowers (Fig. 4e). Cross sections of PinA-1214 anthers

Fig. 3 Gene expression of *uidA* under the control of the wheat *PinA* promoter in transgenic rice during plant development. **a–f** Different views of PinA-1214 seedlings at 3 (**a**, **b**), 6 (**c**, **d**) and 15 (**e**, **f**) DAG. **g–j** Primary (**g**, **i**) and secondary (**h**, **j**) roots of PinA-1214 (**g**, **h**) and Pc-Xb (**i**, **j**) seedlings at 15 DAG. Leaves of 15-day-old (**k**) and 3-month-old (**l**) PinA-1214 plants. Histological analysis of 3 μm sections of PinA-1214 primary (**m**) and secondary (**n**) roots. *Red arrows* highlight strong GUS activity. *Bars*, 100 μm (**m**, **n**)
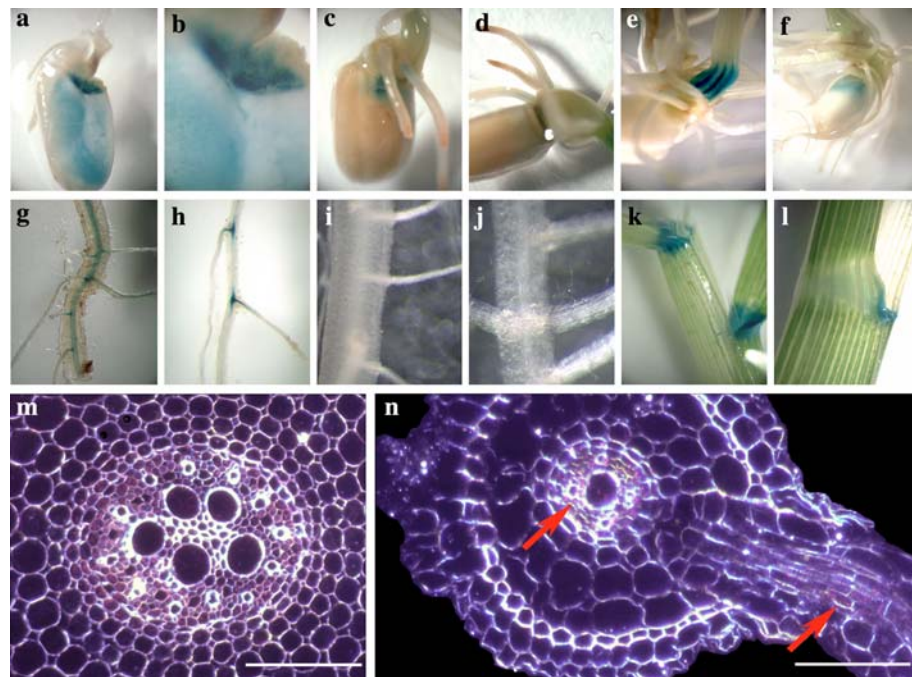
Fig. 4 Gene expression of *uidA* under the control of the wheat *PinA* promoter in transgenic rice flowers. **a** Overview of GUS staining throughout the development of a PinA-1214 flower. **b** Anthers from PinA-1214 flowers of increasing maturity. **c–e** Anthers from PinA-1214 (**c**), PinA-390 (**d**) and PinA-214 (**e**) flowers at the anthesis stage. **f**, **g** Histological analysis of 3 μm sections of PinA-1214 anther (**f**) and palea (**g**). **h** Flower of pC-Xb plant at the anthesis stage. *Bars*, 18 μm (**f**), 180 μm (**g**)
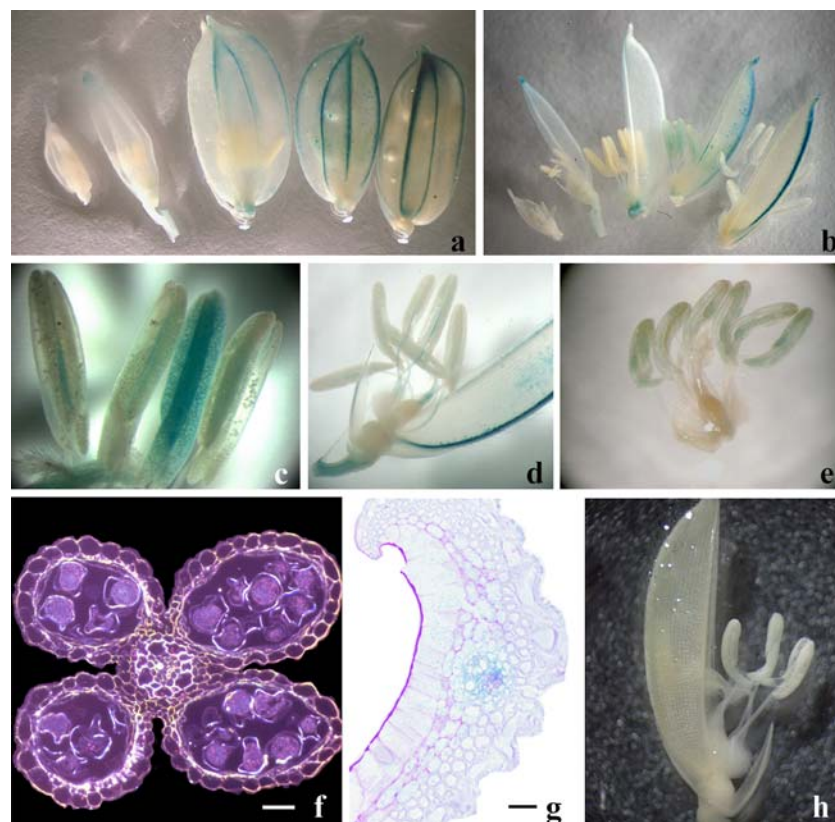
showed that GUS staining was restricted to pollen grains and anther vascular tissues (Fig. 4f). Conversely, in palea of PinA-1214 flowers, GUS staining was only observed in vascular tissues (Fig. 4g). Figure 2h is representative of PinA-136, PinB-1063 and pC-Xb flowers in which no GUS activity was detected.

PinA promoter activity is induced by wounding and by a pathogen in transgenic rice plants

Intense GUS staining was observed at the cutting site of shoot segments made of rolled leaf sheath and blades (Fig. 5a, b), the flower peduncle (Fig. 5c) and
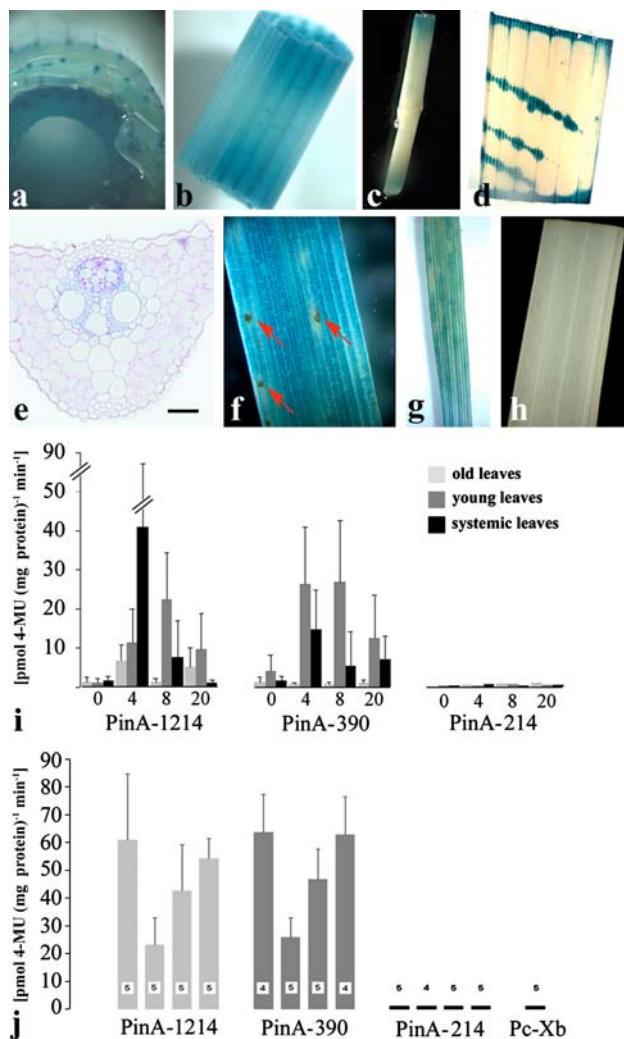
Fig. 5 Gene expression of *uidA* under the control of the wheat *PinA* promoter in transgenic rice leaves after wounding or fungus infection. **a, b** Sectioned leaf blade from a 5-week-old PinA-1214 plant. **c** Flower peduncle of a 6-month-old PinA-1214 plant. **d** Scalpel-wounded leaf sheath of a 3-month-old PinA-1214 plant. **e** Histological analysis of 3 μm sections of wounded PinA-1214 leaf sheath. **f–h** Leaf sheath of PinA-1214 (**f**), PinA-390 (**g**) and pC-Xb (**h**) 72 hpi by *M. grisea*. *Red arrows* mark the positions of hypersensitive reactions observed for PinA-1214 plants. **i** GUS specific activities in protein extracts of wounded leaves harvested at 0, 4, 8 and 20 hfw. Values are means with standard deviation (*n* = 5 plants). **j** GUS specific activities in protein extracts of PinA-1214, PinA-390, PinA-214 and pC-Xb leaves harvested at 36 hpi. Values are means with standard deviation (*n* = 4 or 5 plants for each line). *Bars*, 20 μm (**e**)

stem internodes (data not shown) of PinA-1214 and PinA-390 plants. Leaf sheath and blade sections wounded with a razor blade exhibited deep blue GUS staining only at the wounding sites (Fig. 5d). Light microscopy observation of cross section of wounded leaves showed that GUS staining was preferentially present in vascular bundles (Fig. 5e). These patterns were only observed in samples of PinA-1214 and

PinA-390 transgenic rice plants and no GUS activity was detected in PinA-136, PinB-1063 and pC-Xb plants. All these observations suggest that the *PinA* promoter is induced by mechanical wounding.

In order to confirm this result, we wounded leaves using a multineedle system and quantified GUS activity through a fluorometric assay over a time course period. Young leaves were harvested at 0, 4, 8 and 20 hfw. Data presented in Fig. 5i are mean values of five plants resulting from different transformation events. GUS activity increased as early as at 4 hfw, reached a maximum and then decreased at 20 hfw. This pattern was observed in leaves of PinA-1214 and PinA-390 plants with more intense GUS activity in wounded PinA-390 leaves. The systemic response observed in unwounded PinA-1214 and PinA-390 leaves was highest at 4 hfw. In old leaves, GUS activity was low, especially in those of PinA-390 plants.

To determine whether the expression of the wheat *PinA* gene is enhanced by a pathogen attack, leaves of 4-week-old seedlings of PinA-1214, PinA-390, PinA-214, Pin-A136, PinB-1063 and pC-Xb were inoculated with *M. grisea*, the agent of rice blast. Because the rice cultivar Zhongzuo 321 is rather resistant to this pathogen, we used the most aggressive *M. grisea* strain available (FR13). A limited number of necrotic lesions were visible on infected PinA-1214 leaves (Fig. 5f) resulting from hypersensitive responses, without any lesion indicative of a compatible response. Leaves from infected and control plants were analyzed at 72 hpi. In PinA-1214 (Fig. 5f) and PinA-390 (Fig. 5g) leaves, GUS staining was observed in the entire leaf sheath with more intense staining in stomata guard cells along the vascular bundles. More intense staining was observed in PinA-1214 leaves. GUS staining was not observed in inoculated PinA-214, PinA-136 or PinB-1063 leaves, or in control PinA-1214 leaves sprayed with the gelatin solution (Fig. 5h). GUS quantitative analysis was carried out on PinA-1214, PinA-390 and PinA-214 plants. Strong and similar GUS activity was observed in inoculated PinA-1214 and PinA-390 leaves whereas no GUS activity was detected in inoculated PinA-214 leaves (Fig. 5j). No GUS activity was detected either in control PinA-1214 or PinA-390 leaves, or in inoculated PinB-1063 and pC-Xb leaves. These data confirmed observations of GUS staining.

Two putative wound and/or pathogen responsive sequences displayed DNA binding capacity

Analysis of the 390 bp upstream from the ATG of the *PinA* gene that are sufficient to drive *uidA* expression in response to wounding and pathogen attack showed

sequences related to WUN and TCA *cis*-elements identified in *Brassica oleracea SFR2* gene (Pastuglia et al. 1997) and to two putative *as*-1 like binding sites (Katagiri et al. 1989). The putative WUN box is located at −300, the TCA boxes at −317 and −380 and the *as*-1-like binding sites at −362/−382 (Fig. 1b and Table 1). Because all the wheat *PinA* promoter analysis was carried out in transgenic rice, we verified in wheat that wounding could trigger induction of transcriptional regulators that mediate *PinA* promoter activity. Two short promoter sequences were chosen to test about a putative DNA binding capacity using EMSA. The first *PinA* sequence TACCTAGAAAAA TACAATATCTAATTTCCTCTTGAT contains the putative WUN-box and TCA motifs. The second one AAAAGCAGTGGCTAGAAAGATGACGATAT ATA contains two putative *as*-1-like binding sites in inverted repeat. As shown in Fig. 6, a DNA-protein(s) complex was detected for both motifs at each time point. The protein complex seemed to be more abundant or to display a higher DNA-protein affinity in leaves harvested at 8 hfw. Competitive EMSA experiments were performed using unlabeled derivatives of the same oligonucleotides at an 80-fold molar excess as competitors for complex formation. Addition of unlabeled oligonucleotides almost completely abolished complex formation at each time point (lanes +).

Discussion

We have previously shown that the *PinB* gene is seed specific (Digeon et al. 1999). As a step forward in understanding puroindoline gene regulation, we
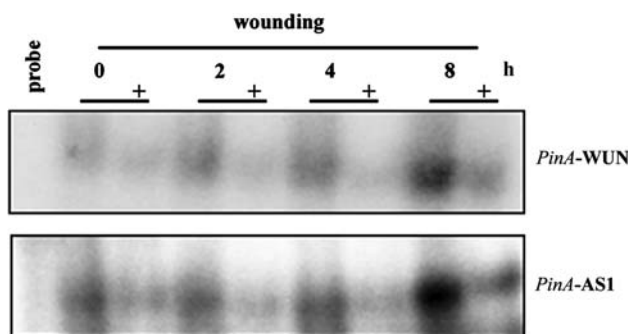
Fig. 6 DNA binding activities of wheat (*Triticum aestivum* L. cv. Chinese Spring) nuclear extracts of wounded leaves on two short *PinA* promoter sequences. Nuclear extracts were prepared from wounded leaves harvested at 0, 2, 4 or 8 hfw. For each nuclear extract, binding reactions were carried out with *PinA*-WUN or *PinA*-AS1 probes, with (+) or without a competitor. Binding reactions without nuclear extracts and without a competitor served as negative control (probe)

isolated a *PinA* gene and examined the temporal and spatial *uidA* gene expression directed by the *PinA* promoter in rice. Serial deletions of the *PinA* promoter were transcriptionally fused to the *uidA* reporter gene and the resulting T-DNA constructs were introduced into rice. In order to compare *PinA* and *PinB* promoter activity in the same context, 1,063 bp of the *PinB* promoter were also fused to the *uidA* reporter gene. Our results showed major differences in the reporter gene expression pattern driven by *PinA* and *PinB* promoters in transgenic rice either in seed, vegetative tissues or in response to wounding and pathogen infection.

PinA and *PinB* promoters exhibit different patterns of activity in transgenic rice seeds

Although PinA-1214 and PinB-1063 promoters drive *uidA* expression in seed, two major differences were observed. First, only the *PinA* regulatory region was found to allow expression of the *uidA* gene in the embryo, and second, expression directed by the *PinA* promoter is more intense within the aleurone layer and epidermis cells whereas the *PinB* promoter directs more concentrated expression in the endosperm. Despite these differences, *PinA* and *PinB* promoters drive *uidA* expression both in endosperm and aleurone cells. Our results do not support those of Dubreil et al. (1998) indicating that PIN-a and PIN-b show different localization, but corroborate those of Capparelli et al. (2005) that clearly showed the co-localization of PIN-a and PIN-b in endosperm and aleurone cells of mature wheat seeds.

During rice seed development, GUS crystals accumulate earlier in PinA-1214 than in PinB-1063 seeds; this is in agreement with the pattern of accumulation of *PinA* and *PinB* gene transcripts reported in wheat seeds (Gautier et al. 1994). During germination, no GUS staining was detected in PinB-1063 seedlings, while PinA-1214 and PinA-390 seedlings displayed GUS staining that is consistent with observation of GUS activity in PinA-1214 and PinA-390 embryos. No *PinA* and *PinB* gene transcript was detected in wheat seedlings (1 to 7-day old) by northern analysis (Gautier et al. 1994). Because RNA extraction was carried out on whole seedlings, too high dilution of gene transcripts and the lack of sensitivity of the method could explain this discrepancy.

In order to identify any short promoter sequences directly involved in gene expression in seeds, the choice of the *PinA* promoter deletions was based on the previous analysis of the *PinB* promoter (Digeon et al. 1999). In rice seed, PinA-1063 and PinA-390 promoter drive *uidA* expression in embryo, endosperm,

aleurone, testa and epidermal cell layers. No GUS accumulation was observed in PinA-214 and PinA-136 seeds indicating that the 214 bp upstream from the ATG of *PinA* gene are not sufficient to confer expression in seeds. For the *PinB* gene, we previously showed that the "210-bp" promoter directs *uidA* expression only in the median zone of the endosperm whereas there is no GUS activity in PinB-124 seeds (Digeon et al. 1999).

In cereals, endosperm expression of genes encoding storage proteins is regulated by the combinatorial interactions of several conserved *cis*-regulatory elements enclosing the prolamin box (P-Box), the AACA, ACGT and GCN4-like (TGA(G/C)TCA) motifs (Morton et al. 1995; Albani et al. 1997; Conlan et al. 1999; Wu et al. 1998, 2000). Sequence alignment of *PinA* and *PinB* promoters (Fig. 1b) shows conservation of the motif TTGAGAAAGG (−204 for *PinA* and −224 for *PinB*) close to the P-Box motif whose consensus sequence is TG(T/A/C)AAA(A/G)(G/T). Several AACA motifs are present at positions −286, −274 and −222 in the *PinA* promoter and at −104 and −340 in the *PinB* promoter. ACGT motifs are found at position −352 (lower strand) and −543 in the *PinA* promoter and at −195 in the *PinB* promoter. Although GCN4-like motifs are not present in the "390-bp" *PinA* and "388-bp" *PinB* promoters that are sufficient to confer *uidA* gene expression in rice endosperm, a GCN4-like motif TGACTCA is present at −992 in the *PinA* promoter and may modulate its expression in endosperm. This analysis suggests that such combinatorial control could play a role in the regulation of puroindoline gene expression in seed and further investigation is required to identify these regulatory mechanisms.

The *PinA* promoter is not seed specific

However, the main difference between the *PinA* and *PinB* gene expression profile is that the *PinA* gene promoter is not seed specific while, as was previously shown (Digeon et al. 1999) and confirmed in this study, the *PinB* gene promoter is seed specific. We indeed showed that the *PinA* gene promoter also drives expression of the *uidA* reporter gene in roots, flowers and leaves. However, it should be noticed that the highest expression was observed in seeds and that the *uidA* expression driven by the *PinA* gene promoter in other organs than seeds was localized in specific tissues. PinA-1214 and PinA-390 transgenic plants displayed a similar GUS staining pattern during rice development suggesting that the "390-bp" fragment is essential for *PinA* gene expression in the whole plant

and not only in seed. In PinA-214 rice, GUS staining was detected only in pollen grains, and no GUS staining was detected in any organs of PinA-136 and control plants.

The *uidA* gene expression pattern driven by the *PinA* promoter in flowers is complex. In PinA-1214, PinA-390 and PinA-214 flowers, GUS staining appears in dehiscent anthers when pollen grains are going to be disseminated. Bearing in mind that puroindolines, the main component of friabilin, are associated with starch granules in endosperm (Greenwell and Schofield 1986; Rahman et al. 1994), this raises two questions: whether this expression pattern is related to starch accumulation in mature pollen grains, and if PIN-a is accumulated in pollen. In addition, in PinA-1214 and PinA-390 flowers, intense GUS staining was observed in the vascular tissues of palea and this expression was spatially and developmentally regulated. Although there is no simple consensus sequence for a "pollen box", analysis of PinA-214 rice indicates that the 214 bp upstream from the ATG may contain specific *cis*-elements that are sufficient for expression in pollen grain. The *PinA* promoter contains two *cis*-elements, AGAAA at −196 and TCCACCAGT at −146 that are closely related to the motifs found in *Lat52* and *Lat59*, two late anther tomato genes (Eyal et al. 1995). The AGAAA motif is also present in the *PinB* promoter at −216 but the second one TGCACCATT at −166 is not so well conserved. Hamilton et al. (1998) described an enhancer element in the maize *ZM13* gene promoter that regulated this gene in *Tradescantia* and maize. Sequence AGGTCA present in the *PinA* promoter at −191 is mutated AGGGAA in *PinB* promoter at −211.

The *uidA* gene expression driven by the *PinA* promoter in roots appears at early stages of rice development and remains constant during plant development with the strongest expression in vascular tissues and in root regions where branching occurs. However, *cis*-elements known to be root- or vascular tissue-specific were not found in the *PinA* promoter fragment we studied. The GUS activity was also observed in programmed abscission zones, i.e. the leaf lamina joint and ligule base. This finding may indicate a role for PIN-a during abscission when the cells form a barrier that protects the exposed surface from desiccation and the entry of parasites. According to the observed induction of *PinA* by the pathogen *M. grisea*, the second role is more likely.

The *PinA* promoter is wound and pathogen inducible

In rice, *uidA* expression driven by the *PinA* promoter was localized in areas of physical disruption or exposed

to pathogen attack. This is obvious in seeds, in which strong expression was observed in aleurone cells, testa, the epidermis and the base of the hypocotyl in germinating seedlings. Likewise, GUS activity was high in pollen grains before expulsion, in the palea protecting the flower and at the abscission zone of stem/leaves at the site where the scar tissues form. Wound induction of the *PinA* promoter was first detected at the cutting edge of leaves during sampling. Moreover, embryos from incised seeds displayed stronger GUS staining than isolated embryos as well as sectioned roots (data not shown). Furthermore, we showed that this induction also occurred in a systemic manner and that *PinA* promoter activity was localized in the vascular bundle, a tissue of choice for propagation of virus and fungus spores.

The Zhongzuo 321 rice cultivar used in this study is highly resistant to the FR13 isolate of *M. grisea* and developed only a few hypersensitive reaction necrotic lesions. At 36 hpi, GUS activity was highly localized in stomata guard cells of the whole leaf suggesting a systemic response. In contrast, no systemic response was observed for the rice *Ltp1* promoter that is also induced by wounding and infection by *M. grisea* (Guiderdoni et al. 2002). Further studies undertaken to compare compatible/incompatible pathogen interactions in rice (with the *PinA* promoter), or in wheat at the mRNA and protein level, could also investigate the specificity of the *PinA* response to pathogen infection. The systemic response of the *PinA* promoter after wounding or inoculation with *M. grisea* suggests the involvement of signaling molecules that still require identification. Plant defense pathways have been widely studied in tobacco, *Arabidopsis* and rice, but little is known about the different signaling pathways in wheat.

We verified by EMSA that the presence in the *PinA* promoter of the putative *cis*-elements related to wounding and/or the pathogen response has a biological significance. A short *PinA* promoter sequence containing the WUN-box and the TCA motif identified in the *B. oleracea SFR2* gene (Pastuglia et al. 1997) forms complex(es) with nuclear extracts isolated from wounded wheat leaves. Identical results were observed with a second *PinA* promoter sequence containing two putative *as*-1 like binding sites (Katagiri et al. 1989) in inverted repeat. TGACG motifs have been shown to bind TGA factors (Lam and Lam 1995) and such factors interact with NPR1 (Després et al. 2000; Zhou et al. 2000) a key regulatory protein that is positioned at the crossroads of multiple defense pathways in plants (for review see Dong 2004; Pieterse and Van-Loon 2004). The strong binding observed at 8 hfw

confirmed the high GUS activity measured in PinA-1213 rice leaves at 8 hfw. In addition, the comparison of *PinA* and *PinB* promoters clearly showed that sequences of putative WUN and TCA elements are not well conserved in the *PinB* promoter, and no putative *as*-1 like binding sites were identified in the *PinB* promoter. These observations are also consistent with the absence of induction of the *PinB* gene promoter in response to wounding and to the pathogen *M. grisea*.

We extended this analysis to putative *cis*-elements whose presence may be related to *PinA* expression in the flower or induction by wounding/pathogen by comparing *PinA* and *PinB* genes isolated from ancestor wheats. All these sequences are conserved within promoters of the *PinA* gene isolated from ancestor wheats and absent or mutated in the *PinB* gene promoter (data not shown). This conservation indicates that the *PinA* gene from related species may have a similar pattern of expression. To verify if this conservation also applies to the related wheat species *Hordeum vulgare*, we aligned sequences of the *PinA* and *hina* (AH014393 reverse complement strand from 128,350 to 127,889) gene promoters. The 500 bp upstream from the ATG displayed 90% sequence identity (data not shown) and the putative *cis*-elements underlined in Fig. 1b and whose presence has been linked either to flower expression or induction due to wounding and or a pathogen attack are very well conserved. This was verified on 14 other *hina* gene sequences (EMBL accession numbers AY644199–AY644212). In contrast, as observed in the wheat *PinB* gene promoter, barley *HOI-B1* and *HOI-B2* gene promoters (Darlington et al. 2001) and other *hinb-1* and *hinb-2* sequences (EMBL accession numbers AY644080–AY644089) do not contain these putative *cis*-elements. This evolutionary evidence implies that the putative *cis*-elements identified in the *PinA* gene promoter are more likely candidates.

Although there is in planta and in vitro evidence supporting the defensive roles of puroindolines against pathogens, this is the first report that the *PinA* gene is induced by wounding or by a pathogen. Expression of puroindolines in transgenic rice enhances disease resistance to fungal pathogens (Krishnamurthy et al. 2001) and expression of wheat PIN-b reduces scab susceptibility in transgenic apple (Faize et al. 2004). Using the maize ubiquitin promoter, overexpression of PIN-b significantly reduced infection symptoms in wheat infected by *Fusarium culmorum* suggesting that puroindolines protect wheat against Fusarium head blight (Gerhardt et al. 2002). Infection of wheat ears was shown to occur mainly during anthesis (Sutton 1982) and affects partially or fully exposed anthers, openings between the lemma and palea of the spikelet/floret

during dehiscence (Lewandowski and Bushnell 2002; Bushnell et al. 2003). In this respect, the *uidA* expression pattern driven by the *PinA* promoter in rice flowers is quite interesting. In vitro, the capacity of PIN-a and PIN-b to inhibit pathogen growth varies considerably depending on the experimental conditions of tests, on pathogens (bacteria or fungi), on the protein concentration and on the synergy between proteins (Dubreil et al. 1998; Capparelli et al. 2005) but in all cases both proteins do display antibacterial and antifungal properties. However, the different *uidA* expression patterns driven by *PinA* and *PinB* promoters and induction of the *PinA* promoter by wounding and by the fungus *M. grisea* suggest a functional difference for each protein at least in terms of physiological situations in which they could be implicated. Both the antimicrobial properties of PIN-a and induction of the *PinA* promoter by wounding and by the pathogen *M. grisea* make this gene a good candidate for biotechnology applications aimed at generating transgenic plants with higher disease resistance.

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