Inducible expression of a fusion gene encoding two proteinase inhibitors leads to insect and pathogen resistance in transgenic rice

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Summary

Plant proteinase inhibitors (PIs) are considered as candidates for increased insect resistance in transgenic plants. Insect adaptation to PI ingestion might, however, compromise the benefits received by transgenic expression of PIs. In this study, the maize proteinase inhibitor (MPI), an inhibitor of insect serine proteinases, and the potato carboxypeptidase inhibitor (PCI) were fused into a single open reading frame and introduced into rice plants. The two PIs were linked using either the processing site of the *Bacillus thuringiensis* Cry1B precursor protein or the 2A sequence from the foot-and-mouth disease virus (FMDV). Expression of each fusion gene was driven by the wound- and pathogen-inducible *mpi* promoter. The *mpi-pci* fusion gene was stably inherited for at least three generations with no penalty on plant phenotype. An important reduction in larval weight of *Chilo suppressalis* fed on *mpi-pci* rice, compared with larvae fed on wild-type plants, was observed. Expression of the *mpi-pci* fusion gene confers resistance to *C. suppressalis* (striped stem borer), one of the most important insect pest of rice. The *mpi-pci* expression systems described may represent a suitable strategy for insect pest control, better than strategies based on the use of single PI genes, by preventing insect adaptive responses. The rice plants expressing the *mpi-pci* fusion gene also showed enhanced resistance to infection by the fungus *Magnaporthe oryzae*, the causal agent of the rice blast disease. Our results illustrate the usefulness of the inducible expression of the *mpi-pci* fusion gene for dual resistance against insects and pathogens in rice plants.

Keywords: *Chilo suppressalis*, Fusion protein, *Magnaporthe oryzae*, Maize proteinase inhibitor, *Oryza sativa*, Potato carboxypeptidase inhibitor.

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world and a source of food for more than half of the world's population. Rice yields can be severely compromised by the lepidopteran insect *Chilo suppressalis* (striped stem borer) (Dale, 1994). Strategies for controlling this pest based on the use of chemical insecticides are not effective for the control of *C. suppressalis* because larvae enter shortly after hatching into the plant and are protected from the effect of insecticide treatment. Moreover, the repeated use of agrochemicals for the control of *C. suppressalis* has several drawbacks, including the lack of specificity, the possible effects on beneficial organisms, the incidence of resistance development upon prolonged application and the adverse impact on human health and the environment.

An alternative approach for the control of *C. suppressalis* is the development of genetically modified rice plants expressing insecticidal genes. Along with this, many crop plants expressing *Bt* genes from the soil bacterium *Bacillus thuringiensis* (encoding δ -endotoxins, also known as Cry proteins) that show insect resistance have been developed (Sanahuja *et al.*, 2011; Stevens *et al.*, 2012). Knowing the ability of insects to develop resistance against insecticidal compounds and that insect control products based on topically applied *Bt* toxins are widely used, it is increasingly clear that alternative or complementary control strategies must be developed to assure that we do not compromise the benefits provided by *Bt*-based insect pest control strategies.

Plant proteinase inhibitors (PIs) have been also identified as candidates for the development of insect-resistant transgenic crops. Plant PIs are usually present in reproductive tissues (seeds, tubers) where they are synthesized and stored (Koiwa *et al.*, 1997; Ryan, 1990). Furthermore, PI gene expression is induced in vegetative tissues by chewing insects and wounding. The usefulness of plant PIs to reduce insect attack in controlled greenhouse conditions and in the field is also documented (Duan *et al.*, 1996; Dunse *et al.*, 2010; Haq *et al.*, 2004; Hilder *et al.*, 1987; Johnson *et al.*, 1989; Stevens *et al.*, 2012; Vila *et al.*, 2005). Moreover, the use of PIs has been regarded as a suitable alternative to *Bt* in pest control, as milder toxicity of these genes is expected to exert a lower selection pressure to the insect.

Plant PIs function in the plant defence response against herbivorous insects via the inhibition of insect digestive proteinases (Broadway, 1996; Johnson *et al.*, 1989; Srinivasan *et al.*, 2005). When plant PIs bind to the target proteinases, they block the digestion of proteins, leading to growth and developmental delays, and increased mortality. However, insects are able to adapt to the presence of inhibitors in the diet by either overproducing PI-sensitive proteinases, replacing the inhibited enzymes by PI-insensitive proteinases, or inducing the production of PI-degrading enzymes (Broadway, 1997; Gatehouse, 2002; Girard *et al.*, 1998a; Giri *et al.*, 1998; Jongsma *et al.*, 1995). A phenomenon of overcompensation to dietary proteinase inhibitors produced in transgenic plants resulting in increased weight gain of the target pest has also been described (Cloutier *et al.*, 2000; De Leo *et al.*, 1998; Girard *et al.*, 1998b). Most of these

problems can be associated with the use of a single PI transgene targeting only one class of proteases in the insect midgut. In this context, the simultaneous expression of PIs with different modes of action in transgenic plants not only might confer increased level of protection against insect attack but also would hinder insect adaptation to PI ingestion.

We previously reported that wounding and insect feeding induce the expression of the *mpi* (maize proteinase inhibitor) gene in maize plants (Cordero et al., 1994). MPI is a bifunctional inhibitor that effectively inhibits digestive serine proteinases, namely elastase and chymotrypsin proteinases from the lepidopteran insects *Spodoptera littoralis* and *Chilo suppressalis* (Tamayo et al., 2000; Vila et al., 2005). In addition to wounding, *mpi* expression is induced by fungal infection in maize plants (Cordero et al., 1994). In this respect, several proteinase inhibitors have been reported to inhibit the *in vitro* growth of phytopathogenic fungi, and, for some of them, a defence role in resistance to fungal pathogens has been proposed (Haq et al., 2004; Kim et al., 2009; Laluk and Mengiste, 2011). However, despite the body of information currently available on plant proteinase inhibitors as insecticidal agents, their potential role as antimicrobial agents remains less explored.

On the other hand, the potato carboxypeptidase inhibitor (PCI) accumulates in potato tubers as well as in wounded leaves of potato plants (Graham and Ryan, 1981; Villanueva et al., 1998). PCI inhibits digestive carboxypeptidase activities from the lepidopteran insect *Helicoverpa armigera* (Bayés et al., 2003). However, the constitutive expression of *pci* in rice has an apparent stimulatory effect on the growth of *C. suppressalis* larvae fed on the *pci* transgenic plants, suggesting an adaptive mechanism in the larval gut to compensate for the effect of PCI ingestion (Quilis et al., 2007).

In this study, we examined the effect of expression of a fusion gene consisting of two plant proteinase inhibitors, *mpi* and *pci*, in transgenic rice focusing on resistance against insect attack and pathogen infection. Two different strategies were assayed for obtaining the fusion gene depending on the sequence used to fuse the two individual PI genes in the form of a single transcriptional unit. In both cases, the expression of the *mpi-pci* fusion gene is driven by the wound- and pathogen-inducible *mpi* promoter. We report that the inducible expression of the *mpi-pci* fusion gene confers resistance to the lepidopteran insect *C. suppressalis* as well as to the fungal pathogen *Magnaporthe oryzae*, the causal agent of the rice blast disease.

Results

Production of transgenic rice plants expressing a *mpi-pci* fusion gene

Two different constructs containing a *mpi-pci* hybrid gene that differed on the linker sequence used to fuse the two genes were prepared (Figure 1a; Methods S1). The first strategy was selected based on the potential of the FMDV2A (foot-and-mouth disease virus 2A) sequence to mediate self-processing of a polyprotein (Halpin et al., 1999). In this way, the MPI and PCI polypeptides were fused into a single open reading frame using the FMVD 2A sequence (hereafter referred to as MPI-2A-PCI protein). The second strategy consisted the use of the nucleotide sequence encoding the processing site of the cry1B precursor protein to fuse the *mpi*- and *pci*-coding sequences (hereafter referred to as MPI-C-PCI protein). In this respect, it is well known that the Cry proteins are synthesised as longer inactive precursor proteins which are proteolytically processed by trypsin-like proteinases in

the insect gut to become an active toxin (Miranda et al., 2001). Knowing the functional importance of the C-terminal tail of PCI for inhibition of carboxypeptidase activities (Marino-Buslje et al., 2000), the *pci* gene was fused in frame and downstream of the *mpi* gene in the two strategies.

The two *mpi-pci* fusion genes were expressed in rice under the control of the *mpi* promoter (−1872/+197 promoter region). This promoter has been shown to be activated in vegetative tissues of transgenic rice in response to mechanical wounding (a plant response to insect feeding) and fungal infection (Breitler et al., 2001; Moreno et al., 2005). The components of the two plant expression vectors used for wound-inducible expression of each *mpi-pci* fusion gene in rice plants are shown in Figure 1a.

Transgenic rice (*O. sativa* elite *japonica* cultivar Ariete) plants were generated by *Agrobacterium*-mediated transformation. Transgene integration in the rice genome was assessed by Southern blot hybridization, most of the plants showing 1 or 2 integration events (Figure S1). The wound-inducible expression of the *mpi-pci* gene in leaves of transgenic rice plants was confirmed through successive generations (T0 to T3 progeny plants) by Northern blot analysis (Figure 1b shows the results obtained on the analysis of T2 homozygous plants). No accumulation of *mpi-pci* transcripts occurred in either wounded leaves from untransformed plants (Figure 1b, WT; see Methods S1 for wounding of plant leaves) or unwounded leaves of transgenic plants (results not shown). Finally, no visible pleiotropic effects on plant growth and development due to the expression of the *mpi-pci* fusion gene were observed (results not shown).

Effect of transgenic expression of the *mpi-pci* fusion gene on insect growth

To determine the effect of transgenic expression of a *mpi-pci* gene on the growth of *C. suppressalis* larvae, a series of feeding trials were carried out. In these experiments, five independent homozygous lines for each fusion gene (*mpi-C-pci* and *mpi-2A-pci* lines) and wild-type plants were assayed (Methods S1). These studies revealed a significant reduction in weight gain in the larvae fed on rice lines expressing one or another *mpi-pci* fusion gene when compared with larvae fed on wild-type plants (Figure 2a). After feeding for 14 days, the average weight of larvae fed on control plants was 38.8 ± 3.1 mg. The weight of larvae fed on *mpi-C-pci* lines was, however, significantly lower than that of larvae fed on control plants, their mean weight ranging from 13.7 to 23.4 mg depending on the line (percentage of weight reduction, 64.6%–39.6%) (Figure 2a). Similarly, larvae fed on *mpi-2A-pci* plants grew significantly slower than those fed on control plants, these larvae reaching a mean weight of 22.1 to 32.5 mg (percentage of weight reduction, 42.9%–16.2%) (Figure 2a). Representative images of larvae recovered at the completion of the 14-day experimental period are shown in Figure 2b. On the basis of the weight of larvae recovered in these feeding experiments, the larval population was distributed into different groups. Whereas larvae recovered from wild-type plants distributed along the various weight classes and reached the higher weight classes (>70 mg), most of the larvae recovered from *mpi-pci* lines remained in the lower weight classes, and none of the larvae recovered from these plants reached the 70 mg weight mark (Figure S2). Together, feeding trials established that larvae fed on rice plants expressing a *mpi-pci* gene under a wound-inducible regime showed an important reduction in their weight when compared with that of larvae fed on control plants.

(a) Vector 1: *pC1300::mpi prom::mpi-2A-pci::mpi ter* (b) Vector 2: *pC1300::mpi prom::mpi-C-pci::mpi ter*

Figure 1 Expression of the *mpi-pci* fusion gene in transgenic rice plants. (a) Schematic representation of vectors used for rice transformation. Vector 1 contained the *mpi-2A-pci* fusion gene, whereas vector 2 contained the *mpi-C-pci* fusion gene. The wound-inducible *mpi* promoter was used to drive the expression of the *mpi-pci* fusion gene. The *hptII* gene encoding resistance to hygromycin served as the selectable marker in rice transformation. The regions used as probe for genomic Southern blot analyses (Figure S1) are indicated with solid lines. (b) Northern blot analysis of total RNA isolated from wounded leaves of transgenic and control untransformed (WT, Ariete) rice plants. Representative lines transformed with the *mpi-2A-pci* or the *mpi-C-pci* fusion gene (T2 generation) are shown. Hybridization to the 18S ribosomal RNA is shown in the lower panel as a control for loading.

Wound-inducible expression of the *mpi-pci* fusion gene in rice confers insect resistance

Knowing that rice plants expressing a *mpi-pci* fusion gene had a negative impact on larval growth, we assessed the performance of rice plants during infestation with *C. suppressalis* larvae under controlled greenhouse conditions. Three weeks after infestation, transgenic plants, either the *mpi-C-pci* or the *mpi-2A-pci* lines, showed normal development, whereas control plants were severely damaged or dead (Figure 3a). Furthermore, Northern blot analysis revealed important levels of *mpi-pci* transcript accumulation in leaves of all the transgenic lines used in these bioassays, this observation indicating that the *mpi* promoter directs high level of transgene expression in *C. suppressalis*-infested rice plants (Figure 3b). This is in agreement with the results previously reported on the *mpi* promoter activity in transgenic rice (Breitler *et al.*, 2001, 2004; Vila *et al.*, 2005).

It is well known that infestation by *C. suppressalis* leads to the formation of empty panicles, a condition commonly called 'white head'. Accordingly, we determined the percentage of white panicles in rice plants that have been infested with *C. suppressalis* larvae. For this, a subset of noninfested and *C. suppressalis*-infested transgenic and wild-type plants were allowed to continue growth and further analysed for production of white panicles (Figure 3c). Whereas the percentage of white panicles in wild-type plants was 20.5%, the transgenic lines produced a percentage of white panicles ranging from 5.9% to 12.5% in the *mpi-C-pci* lines or from 7.7% to 13.6% in the *mpi-2A-pci* lines.

Collectively, results obtained in insect bioassays revealed that the expression of the *mpi-pci* fusion gene in rice plants confers protection against the lepidopteran insect *C. suppressalis*. In addition to prevent plant damage caused by *C. suppressalis*, rice plants expressing a *mpi-pci* gene produced less white panicles than wild-type plants.

Analysis of transgene-derived protein products

Immunoblot experiments were carried out to examine the accumulation of the transgene products in rice tissues in each strategy assayed in this work. For this, protein extracts were prepared from wounded leaves of wild-type and transgenic plants, both *mpi-C-pci* and *mpi-2A-pci* plants (homozygous T2 generation), and probed with an anti-MPI antibody raised against the pure MPI protein (Tamayo *et al.*, 2000) (for details on Western blot analysis see Methods S1). As a control, protein extracts from wounded leaves of rice plants expressing the *mpi* gene under the control of its own promoter (Vila *et al.*, 2005) were analysed. As expected, the MPI polypeptide accumulated in *mpi* rice plants (Figure 4a). Immunoblot analysis of protein extracts from *mpi-C-pci* lines also confirmed the accumulation of the fusion protein, which was absent in wild-type plants (Figure 4a). No immunological reactions occurred with the protein extracts from rice plants when the pre-immune serum was used (results not shown).

The expression strategy using the 2A sequence as the linker peptide aimed to produce the two individual proteinase inhibitor proteins, MPI and PCI, from the hybrid protein in rice tissues. Immunoblot analysis of protein extracts obtained

Figure 2 Insect feeding bioassays. (a) Growth of *Chilo suppressalis* fed on control wild-type (WT, white bar), *mpi-C-pci* (grey bars) and *mpi-2A-pci* (black bars) plants (homozygous T2 lines). Fourteen days after infestation with L2 *C. suppressalis* larvae, plants were dissected and larvae were recovered and individually weighted. The percentage of weight reduction in larvae fed on each transgenic line relative to larvae recovered from control plants is indicated at the bottom. Data sets were analysed for significant differences using Student's t-test (** $P \leq 0.001$; * $P \leq 0.05$). Error bars indicate the standard deviation of the error (SE). The distribution of the larval population according to their weight for each line is presented in Figure S2. (b) Representative *C. suppressalis* larvae recovered from wild-type (WT), *mpi-C-pci* (line 6) or *mpi-2A-pci* (line 38) rice plants. Bar = 0.5 cm

from the *mpi-2A-pci* lines revealed accumulation of both, the fusion uncleaved MPI-2A-PCI protein and the MPI protein, indicating that the fusion protein was partially processed in the rice leaves (Figure 4a). The cleavage efficiency varied among the different transgenic lines. By comparing the band intensities with those of known amounts of MPI protein, the MPI content in *mpi-2A-pci* lines was estimated to be 0.10–0.25% of total soluble proteins (% of MPI in total soluble protein) depending on the transgenic line. Longer exposure times of immunoblots containing protein extracts from *mpi-2A-pci* plants indicated that the MPI-2A-PCI polypeptide was properly cleaved as revealed by the absence of MPI-containing truncated protein signals (Figure S3).

During the course of this work, the pure PCI protein was used to raise polyclonal antibodies in either rabbits or egg yolk which were then used for immunoblot analysis of protein extracts from rice leaves. Unfortunately, these analyses did not allowed us to detect the PCI polypeptide in the same protein extracts in which MPI was detected. Detection problems arising from inefficient extraction of PCI from plant tissues, or low antigenicity of the PCI polypeptide, might explain these results. In favour of the low antigenicity of PCI, pure PCI polypeptide was undetectable by Western blot analysis (data not shown).

Failure to detect PCI in rice protein extracts prompted us to examine PCI activity in leaves of *mpi-pci* rice plants. For this, total protein extracts were prepared from wounded leaves of either *mpi-C-pci* or *mpi-2A-pci* plants which were then subjected to affinity chromatography on immobilized carboxypeptidase A (CPA). As controls, pure PCI protein as well as protein extracts from leaves of rice plants constitutively expressing *pci* (Quilis *et al.*, 2007) was subjected to affinity chromatography. In all the cases, the eluted fractions were assayed for their ability to inhibit bovine CPA activity. When pure PCI protein or protein extracts from *pci* rice plants were analysed, the inhibitory activity of PCI against CPA was recorded in fractions eluted at pH 11.0, but not in protein fractions eluted at pH 9.0 (Figure 4b, upper panels). Equally, when protein extracts from *mpi-C-pci* or *mpi-2A-pci* plants were subjected to affinity chromatography, the protein fractions eluted at pH 11.0 showed inhibitory activity against CPA (Figure 4b, lower panels). A certain level of CPA inhibition in the first fraction eluted at pH 9.0 (*mpi-C-pci* plants) was also detected, pointing to a weaker interaction of the fusion protein with CPA during affinity chromatography relative to that of the PCI alone.

Together, these results suggest that the two *mpi-pci* fusion genes are efficiently transcribed and translated to produce a MPI-

Figure 3 Resistance to *C. suppressalis* of rice plants expressing a *mpi-pci* fusion gene. (a) Phenotype of wild-type (WT) and *mpi-pci* plants that had been infested with *C. suppressalis* larvae. Bioassays for insect resistance were performed with *mpi-C-pci* (lines 4, 6, 7, 16 and 18) and *mpi-2A-pci* (lines 7, 17, 24, 25 and 38) plants. Results for the *mpi-C-pci* line 7 and the *mpi-2A-pci* line 7 are shown (similar results were obtained for the other lines here assayed for each gene). (b) Northern blot analysis of RNAs obtained from *mpi-C-pci* or *mpi-2A-pci* rice plants used in bioassay experiments. Leaves from at least 6 plants for each line were pooled and analysed by Northern blot (20 µg of total RNA each sample). Ethidium bromide staining served as loading controls. (c) Percentage of white panicles in rice plants infested with *C. suppressalis* larvae relative to the total panicles obtained for each line. Data shown are from one of two experiments that produced similar results.

PCI fusion protein when fused by either the FMDV 2A sequence or the processing sequence of the Cry1B precursor protein. Concerning the *mpi-2A-pci* rice plants, processing of the fusion protein and accumulation of the MPI polypeptide in rice tissues was demonstrated. Limitations for immunodetection of PCI by Western blot analysis hampered detection of the PCI polypeptide produced by cleavage of the MPI-2A-PCI fusion protein in rice tissues. Even though the accumulation of the PCI polypeptide plants could not be clarified in this work, we have shown that protein extracts from *mpi-C-pci* plants exhibited inhibitory activity against CPA, supporting that the PCI domain of the fusion protein is active and effective for inhibition of carboxypeptidase activity.

Resistance of *mpi-pci* rice plants to infection by the rice blast fungus *Magnaporthe oryzae*

In this work, the functional relevance of transgenic expression of the *mpi-pci* fusion gene in terms of resistance to infection by *M. oryzae* was examined. Results previously reported by our group indicated that rice plants constitutively expressing the *pci* gene exhibit enhanced resistance to infection by this fungus (Quilis *et al.*, 2007). Accordingly, resistance to fungal infection of *mpi-pci* rice plants was determined and compared to that of *pci* rice plants (Methods S1). For this, leaves of the transgenic plants were locally

inoculated with spores from a *gfp*-expressing *M. oryzae* isolate as previously described (Campos-Soriano and San Segundo, 2009). Differences in the degree of disease symptoms caused by *M. oryzae* between *mpi-pci* lines and wild-type plants were clearly observed. At 3 days after inoculation, small lesions were generally observed in leaves of the *mpi-pci* and *pci* lines, whereas the leaves from control plants were visibly damaged under the same experimental conditions (Figure 5a). Fluorescence microscopy revealed the presence of a fluorescent hyphae growing on the leaf surface of wild-type plants (Figure 5a).

Finally, image analysis was used to determine the percentage of leaf area affected by blast lesions in *mpi-pci* and *pci* plants at 6 days postinoculation. In agreement with the visual inspection, the inoculated leaves from *mpi-pci* and *pci* transgenic lines exhibited a lower percentage of diseased area relative to inoculated leaves from wild-type plants (Figure 5b). Generally, the rice lines expressing the *mpi-C-pci* gene exhibited relatively reduced blast disease symptoms compared to the transgenic lines expressing either the *mpi-2A-pci* gene or the *pci* gene alone.

Together, disease resistance assays demonstrated that expression of the *mpi-pci* fusion gene in rice, either the *mpi-2A-pci* or the *mpi-C-pci* gene, confers enhanced resistance against the rice blast fungus *M. oryzae*. Furthermore, these results indicated that the

Figure 4 Protein analysis of transgenic plants expressing a *mpi-pci* fusion gene. (a) Immunodetection of MPI in total protein extracts obtained from leaves of wild-type (WT) plants and plants harbouring the *mpi* gene (Vila *et al.*, 2005), the *mpi-C-pci* (lines 4 and 6) or the *mpi-2A-pci* (lines 38 and 7). In all the cases, transgene expression was driven by the wound-inducible *mpi* promoter. Leaves were mechanically wounded and harvested at 16 h after wounding. Protein extracts (40 µg each extract) were separated by 15% SDS-PAGE and probed with an anti-MPI antibody (Tamayo *et al.*, 2000). (b) Affinity chromatography on immobilized CPA of pure PCI (Sigma) and protein extracts from rice plants constitutively expressing *pci* (line 7.34), *mpi-C-pci* plants (line 7) and *mpi-2A-pci* (line 7) plants. Eluted fractions were analysed for inhibition of bovine CPA activity using AAFP as the substrate (Quilis *et al.*, 2007). Lanes E1a to E1c, fractions eluted using buffer at pH 9.0. Lanes E2a to E2c, fractions eluted using buffer at pH 11.0. In each sample, CPA activity is referred to the activity of the enzyme in the corresponding buffer (considered as 100%).

MPI-C-PCI fusion protein might be effective for inhibition of fungal proteinases (as it was previously demonstrated for PCI; Quilis *et al.*, 2007). Knowing that the fusion protein is partially processed in rice leaves of *mpi-2A-pci* (see Figure 4a), the contribution to the phenotype of disease resistance that is observed in these plants might be due to the activity of the MPI-2A-PCI protein and the PCI protein originating from the fusion protein.

Discussion

In this work, we show that the expression of a fusion gene encoding two proteinase inhibitors, namely the MPI and PCI inhibitors, in transgenic rice provides dual resistance against insect attack and pathogen infection. The protective effect of the fusion gene was achieved when using either the FMDV2A linker sequence or the processing region of the Cry1B precursor protein to fuse the two proteinase inhibitor polypeptides. Although evidence exists in the literature on the insecticidal properties of plant PIs, very limited information is currently available about a dual protective effect against insects and pathogens in transgenic crops expressing plant PIs. Only, the simultaneous expression of a trypsin inhibitor (sporamin) gene and a phytocystatin gene in transgenic tobacco plants was reported to be effective for resistance against insect and pathogen attack (Senthilkumar *et al.*, 2010). More recently, the UNUSUAL SERINE PROTEASE INHIBITOR (UPI) gene was reported to function in resistance to

necrotrophic fungi and insect herbivory in Arabidopsis plants (Laluk and Mengiste, 2011).

The transgenic rice expressing a *mpi-pci* fusion gene under the control of the *mpi* promoter showed normal growth and development. In other studies, the constitutive expression of proteinase inhibitor genes in transgenic plants has been shown to have a negative impact on plant development (Zavala *et al.*, 2004). Clearly, the use of a wound- and pathogen-inducible *mpi* promoter (instead of a constitutive promoter) minimizes the possibility of alterations in fitness of the transgenic plants while driving levels of transgene expression sufficient to confer protection to the plant. An additional advantage of using the *mpi* promoter is that this promoter is not active in pollen, seed embryo or endosperm of rice plants, thus reducing food and environmental concerns (Breitler *et al.*, 2001, 2004). Together, the results here presented suggest that the *mpi* promoter can be considered a good candidate to drive conditional expression of insecticidal and antifungal genes in transgenic rice.

Insect feeding experiments revealed that the weight gain of *C. suppressalis* larvae fed on *mpi-pci* plants was reduced compared to that of larvae fed on wild-type plants. Based on the inhibitory properties of MPI and PCI (MPI inhibits insect elastase and chymotrypsin activities, whereas PCI inhibits insect carboxypeptidases), a broad spectrum of insect digestive proteinases is expected to be affected by the coordinated and complementary activity of the two inhibitors. This fact might well explain

Figure 5 Resistance of rice plants expressing the *mpi-pci* gene to *M. oryzae*. (a) Leaves from transgenic rice lines harbouring the *mpi-C-pci*, *mpi-2A-pci*, *pci* or wild-type (WT) plants were inoculated with spores of the *gfp*-expressing *M. oryzae* isolate (10^6 spores/mL) as previously described (Campos-Soriano *et al.*, 2013). Disease symptoms were observed by light and fluorescent microscopy at 3 days after inoculation. Representative results from one of three experiments that produced similar results are shown. (b) Percentage of leaf area affected by blast lesions was determined using the image analysis software Assess v. 2.0 for plant disease quantification at 6 days after inoculation with *M. oryzae* spores. Data sets were analysed for significant differences using Student's *t*-test ($*P \leq 0.1$). Results are shown as mean values $\pm$ SE.

the observed effectiveness of this strategy in conferring insect resistance. The mean weight reduction in larvae fed on rice plants expressing a fusion gene was 39.6%–64.6% and 16.2%–42.9% for the *mpi-C-pci* and *mpi-2A-pci* lines, respectively. In this respect, we previously reported that transgenic expression of the *mpi* gene alone (also under the control of its own promoter) results in a mean weight reduction in *C. suppressalis* larvae of 23.0%–28.6% depending on the line (Vila *et al.*, 2005). Further studies are, however, needed to conclusively determine whether transgenic expression of the *mpi-pci* fusion gene is more effective for inhibition of larval growth than the expression of the *mpi* gene alone.

On the other hand, results previously reported by our group indicated that production of the PCI inhibitor alone in the rice plant might be counterproductive in terms of insect resistance, as the weight gain of *C. suppressalis* larvae fed on rice plants constitutively expressing the *pci* gene was significantly larger than that of larvae fed on wild-type plants (Quilis *et al.*, 2007). It can then be hypothesized that the production of the two plant inhibitors, MPI and PCI, compensates the negative effects that occur in *C. suppressalis* larvae fed on rice plants expressing the *pci* gene alone. If so, the simultaneous production of the PCI and MPI inhibitors in rice would counteract adaptive mechanisms in the complement of digestive proteinases and minimize the compensatory response of *C. suppressalis* larvae after ingestion of the PCI alone.

Concerning the mechanisms underlying insect resistance in *mpi-pci* rice plants, two scenarios can be envisaged. Firstly, there

is the possibility that the MPI-PCI fusion protein acts as a multifunctional proteinase inhibitor. In this respect, efforts have been made to obtain multifunctional proteinase inhibitors exhibiting activity against multiple target proteinases (Schluter *et al.*, 2010). Such approaches involve chimeric proteins that integrate complete or partial functionally significant regions of the inhibitor sequences with a broader inhibitory range against target proteinases (Brunelle *et al.*, 2005; Inanaga *et al.*, 2001; Outchkourov *et al.*, 2004). Most of these studies, however, have been approached by *in vitro* inhibition assays on insect gut protein extracts or by insect feeding assays using artificial diets supplemented with isolated PIs. Secondly, the activity of the two partners originating from the fusion protein either in the rice tissue (*mpi-2A-pci* plants) or in the gut of *C. suppressalis* larvae (*mpi-C-pci* plants) would be responsible for the observed phenotype of resistance. In this respect, whereas the *in vivo* processing of the MPI-2A-PCI fusion protein in rice leaves has been confirmed in this work, the proteolytic processing of the fusion protein in the insect gut has not been demonstrated yet. Further analysis will aid in determining whether insect resistance in *mpi-C-pci* and *mpi-2A-pci* rice plants is mediated by the MPI-PCI fusion protein acting as a multifunctional proteinase inhibitor or by the activity of the two independent inhibitors.

As for the PCI inhibitor, it is well known that the C-tail residues of the PCI polypeptide are essential for enzyme inhibition (Marino-Buslje *et al.*, 2000). There is then a possibility that the PCI protein, either alone or in the form of uncleaved MPI-PCI fusion protein, exerts its inhibitory activity against carboxypepti-

dase activities. The observation that protein extracts from *mpi-C-pci* plants exhibit inhibitory properties against carboxypeptidase activity, together with the observed phenotype of blast disease resistance in these plants, favours the possibility that the PCI domain of the MPI-C-PCI fusion protein is functional and active for inhibition of target carboxypeptidases. Then, fusing the PCI polypeptide to the C-terminus region of proteins of interest in the form of polyproteins might then be a suitable strategy to obtain insect and/or pathogen resistance in transgenic plants.

Moreover, we show that expression of a *mpi-pci* fusion gene in rice does not confer levels of protection against *M. oryzae* significantly higher than those occurring in the rice plants expressing the *pci* gene alone. Contrary to the *pci* rice plants, the rice plants expressing the *mpi* gene alone do not show resistance to infection by the rice blast fungus *M. oryzae* (results not shown). From these observations, it can be reasoned that the protective effect that is observed in *mpi-C-pci* and *mpi-2A-pci* plants results from the antifungal properties of PCI (Quilis et al., 2007). If so, the two inhibitors would not act in a synergistic manner in conferring resistance to *M. oryzae* infection in transgenic rice.

Concerning the strategy based on the use of the FMVD2A sequence, it should be here mentioned that this strategy has been used for co-expression of different genes in transgenic plants. Some examples are the expression of reporter genes in tobacco (de Felipe et al., 2006; Halpin et al., 1999; Ma and Mitra, 2002), the production of lignocelluloses degradation enzymes or self-cleaving antimicrobial polyproteins in tobacco or Arabidopsis plants (François et al., 2004; Lee et al., 2012), or coexpression of carotenoid or tyramine derivatives biosynthetic enzymes in the rice endosperm (Park et al., 2009; Ralley et al., 2004). However, when using the FMVD2A sequence to fuse two antimicrobial proteins (snaking and defensin) in the form of a hybrid protein, no cleavage products were detected in tobacco and potato plants (Kovalskaya et al., 2011). Our *in vivo* experiments demonstrated a clear phenotype of resistance to insect attack and pathogen infection in the *mpi-2A-pci* rice plants, despite the lack of complete processing of the *in planta*-produced fusion protein. At present, the contribution of the fusion MPI-2A-PCI protein and the individual proteins derived from the fusion protein (MPI and PCI) to the observed phenotype of resistance against *C. suppressalis* remains unknown. Altogether, these findings support the need of addressing on a case-by-case basis the evaluation of the FMVD2A sequence as a linker peptide to fuse proteins of interest in a particular plant species.

An important advantage of the strategy assayed in this work is that a single transformation event can lead to the production of plant PIs with different modes of action using only a single promoter, thus avoiding the risk of homology-based silencing, transgene segregation along successive generation or the need of different promoters or selectable markers (as it occurs in other strategies for co-expression of multiple transgenes). Clearly, different approaches can be used for the simultaneous expression of insecticidal and/or antifungal genes in plants, each one having its own advantages and drawbacks to be adopted in crop biotechnology. For instance, the integration of multiple transgenes in a single plant can be achieved by either crossing individual transformants, by sequential transformation, and by co-transformation either with a single plasmid equipped with multicassettes or with multiple plasmids can be approached. Such strategies are, however, time-consuming and require the use of different selectable marker genes. For single-plasmid co-transformation,

the main technical limitation is the difficulty to assemble complex plasmids with multiple gene cassettes, whereas the success of co-transformation with multiple plasmids depends on the frequency by which independent transgenes are integrated into the host genome (transgenes can also segregate in successive generations). Expression of multiple genes in a single transgenic plant also requires the use of different promoters to avoid homology-dependent transgene silencing. Here, it is worthy to mention that there are few reports on insect resistance based on the co-expression of multiple proteinase inhibitors in transgenic plants. Gene pyramiding for insect resistance using plant PIs has been reported by crossing transgenic lines expressing individual genes or using a plant transformation vector that harbours a double expression cassette (Abdeen et al., 2005; Dunse et al., 2010).

Due to the focus on agricultural importance, expression of genes encoding multiple insecticidal factors appears to be critical for long-term control of insect pests. Knowing that the two components of the MPI-PCI fusion protein are effective for inhibition of insect proteinases with different mechanisms of action (quimotrypsin and elastase by MPI, carboxypeptidases by PCI), the simultaneous expression of these plant proteinase inhibitors is expected to maintain its insecticidal properties longer than inhibitors acting on proteinases with a single mechanistic mode of action by hindering insect adaptation to proteinase inhibitor ingestion. The use of *mpi-pci* fusion genes, either alone or adjunct to Bt, can thus be considered as a promising strategy for the long-term protection of rice plants against the striped stem borer *C. suppressalis*. As an additional advantage, the transgenic expression of the *mpi-pci* fusion gene in rice confers protection against the rice blast fungus *M. oryzae*. This fungus is the causal agent of the rice blast disease, one the most devastating fungal disease of cultivated rice worldwide (Talbot, 2003). Currently, the control of the rice blast disease depends on the use of chemically synthesized fungicides. Along with this, the *mpi-pci* rice plants represent an alternative to the economically costly and environmentally undesirable chemical control of the rice blast disease. The ultimate goal of insect and pathogen resistance of the rice plants expressing a *mpi-pci* fusion gene needs now to be confirmed by further evaluation in the field.

Experimental procedures

Construction of plant expression vectors and rice transformation

Two vectors containing a *mpi-pci* fusion gene were prepared in this work and used for rice transformation (Figure 1a, Table S1). In both cases, the coding sequence of the *pci* gene was fused in frame and downstream of the *mpi* gene through either the FMVD2A linker sequence (*mpi-2A-pci* gene) or the cleavage site of the Cry1B precursor protein (*mpi-C-pci* gene). Expression of each fusion gene is driven by the *mpi* promoter and terminator regions (Moreno et al., 2005). Transformation was carried out using the Mediterranean elite *japonica* rice (*Oryza sativa* L.) cultivar Ariete. Details on preparation of expression vectors and production of transgenic rice plants by *Agrobacterium*-mediated transformation of embryogenic calli derived from mature embryos are indicated in Supporting Information. T₀ plants were transferred to a containment glasshouse and examined for transgene expression and integration. Homozygous transgenic lines were identified in the T₂ generation. All rice plants were grown at 27 ± 2 °C under 18-h/6-h light/dark photoperiod.

RNA and DNA analyses

Total RNA was isolated from the leaves of transgenic and control plants using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). For Northern blot analysis, RNAs were subjected to formaldehyde-containing agarose gel electrophoresis and transferred to Hybond-N membranes (Amersham Biosciences, Piscataway, NJ). A DNA fragment coding for the specific *mpi-pci* fusion gene (*mpi-2A-pci* or *mpi-C-pci*) was used as a probe. All probes were gel-purified and radioactively labelled by random priming, according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Hybridizations were conducted at 42 °C in 40% formamide in the hybridization solution.

Genomic DNA was extracted using MATAB (mixed alkyl-trimethylammonium bromide) as the extraction buffer (Coca *et al.*, 2004). For Southern blot analysis, genomic DNA was digested with KpnI restriction enzyme, electrophoresed on 0.8% agarose gels, transferred onto nylon membranes (Hybond-N, Amersham, UK) and hybridized to DNA probes ³²P-labelled by random priming (Roche Diagnostics GmbH, Mannheim, Germany). Hybridizations were carried out at 65 °C (Coca *et al.*, 2004).

Preparation of protein extracts and immunoblotting

Protein extracts were prepared from wounded leaves of transgenic and nontransformed plants. Plant material was ground with liquid nitrogen, and total proteins were extracted using 50 mM Tris-HCl, pH 8, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol and 0.1% (v/v) Triton X-100 as the extraction buffer. Extractions were carried out at 4 °C for 2 h with continuous slow stirring. Protein quantification was performed using Bio-Rad protein assay reagents (Bio-Rad, Madrid, Spain). Immunoblots were prepared as indicated in Supporting Information. To determine the level of MPI accumulation in total protein extracts from leaves of *mpi-2A-pci* plants, the MPI protein was expressed in *Escherichia coli* (Tamayo *et al.*, 2000), and different dilutions of MPI protein were applied to each blot as standards. The MPI levels were measured from digitalized images of the blots (Quantity One Program from Bio-Rad).

Carboxypeptidase A affinity chromatography and activity assays

Total protein extracts from transgenic plants were fractionated by affinity chromatography using a column of immobilized bovine CPA (CPA-agarose, Sigma, St Louis, MO, USA). The column was washed successively with 2.5 mL of 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl; 50 mM Tris-HCl, pH 9.0; and 50 mM Na₂HPO₄, pH 11.0. Eluted fractions (0.5 mL) were collected with each buffer and neutralized to pH 7.5.

To determine the inhibitory activity of eluted fractions, 95 µL of each fraction was pre-incubated with 5 µL of 0.5 µM bovine CPA (Sigma) for 10 min on ice. Then, CPA activity was assayed using *N*-(4-methoxyphenyl-azoformyl)-L-phenylalanine (AAFP, Bachem, Bubendorf, Switzerland) at a final concentration of 0.05 mM (Quilis *et al.*, 2007). The final volume of 200 µL was added to a 96-well plates, and CPA activity was determined by decreasing absorbance at 350 nm over time. As positive control of inhibition, CPA activity was determined using different concentrations of commercial PCI (Sigma). For each fraction, the relative CPA activity was calculated as comparison of the CPA activity after addition of the corresponding elution buffer.

Insect bioassays of transgenic plants

Larvae of *C. suppressalis* were obtained from rice fields in the Camargue (France) and Delta del Ebro (Spain). Feeding assays with *C. suppressalis* larvae were performed as described by Vila and co-workers (Vila *et al.*, 2005). Further experimental details can be found in Supporting Information. Resistance of transgenic rice plants to *C. suppressalis* was performed as described above, and plant symptoms (white panicles) were recorded after 21 days postinfestation.

Blast resistance assays

Resistance of *mpi-pci* and *pci* rice plants to infection by the rice blast fungus *M. oryzae* was carried out using the detached leaf assay (Coca *et al.*, 2004) using a *gfp*-expressing *M. oryzae* isolate (*gfp*-PR9) (Campos-Soriano and San Segundo, 2009; Campos-Soriano *et al.*, 2013). Further experimental details can be found in Supporting Information.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Southern blot analysis of the *mpi-pci* transgenic lines (T0 generation).

Figure S2 Growth of *Chilo suppressalis* larvae fed on transgenic rice plants (T2 homozygous generation) expressing a *mpi-pci* fusion gene under the control of the *mpi* promoter.

Figure S3 Immunodetection of the MPI protein in rice plants expressing the *mpi-2A-pci* gene under the control of the *mpi* promoter.

Table S1 Oligonucleotide primer sequences used in this study.

Methods S1 Construction of plant expression vectors, rice transformation, wounding of plant leaves, immunoblot analysis, insect bioassays and blast resistance assays.