The role of thionins in rice defence against root pathogens

HONGLI JI^{1,2}, GODELIEVE GHEYSEN¹, CHHANA ULLAH¹, RUBEN VERBEEK^{1,3}, CHENJING SHANG¹, DAVID DE VLEESSCHAUWER³, MONICA HÖFTE³ AND TINA KYNDT^{1,*}

¹Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, B-9000 Ghent, Belgium

²Institute of Plant Protection, Sichuan Academy of Agricultural Sciences, Chengdu 610066, China

³Laboratory of Phytopathology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, B-9000 Ghent, Belgium

SUMMARY

Thionins are antimicrobial peptides that are involved in plant defence. Here, we present an in-depth analysis of the role of rice thionin genes in defence responses against two root pathogens: the root-knot nematode *Meloidogyne graminicola* and the oomycete *Pythium graminicola*. The expression of rice thionin genes was observed to be differentially regulated by defence-related hormones, whereas all analysed genes were consistently down-regulated in *M. graminicola*-induced galls, at least until 7 days post-inoculation (dpi). Transgenic lines of *Oryza sativa* cv. Nipponbare overproducing *OstH17* revealed decreased susceptibility to *M. graminicola* infection and *P. graminicola* colonization. Taken together, these results demonstrate the role of rice thionin genes in defence against two of the most damaging root pathogens attacking rice.

Keywords: defence, *Meloidogyne graminicola*, overexpression, *Pythium graminicola*, qRT-PCR, rice thionins.

INTRODUCTION

In nature, plants are attacked by a diverse range of pathogens and herbivores, which cause major yield reduction. However, plants have evolved sophisticated strategies to protect themselves from disease and herbivore feeding. As soon as a pathogen is recognized, plants initiate rapid defence responses, activating signalling pathways and accumulating pathogenesis-related (PR) proteins (Durrant and Dong, 2004; Fritig *et al.*, 1998; Goodman and Novacky, 1994). To date, at least 17 distinct families of PR proteins have been recognized on the basis of their serological relatedness, biological activity and amino acid sequences (Van Loon *et al.*, 2006). In rice, only *PR1*, *PR8* and *PR10* have been reported to be consistently induced following bacterial and fungal infection (Kim *et al.*, 2008; Mitsuhashi *et al.*, 2008; Park *et al.*, 2004). Other PR genes are regulated differentially depending on the pathogen species (Chen and Ronald, 2011). Some of these PR proteins are of low molecular weight, have antimicrobial activity and are highly

resistant to proteases. Among them, the PR13 family, also known as thionins, is a group of antimicrobial peptides. They are generally 45–47 amino acids long, usually basic and cysteine rich, and have a molecular weight of about 5 kDa. Thionins are synthesized as much larger preproteins, including a signal peptide, a basic mature thionin domain and a long acidic domain. The six to eight cysteines in a mature thionin form disulfide bridges (Bohlmann and Apel, 1991; Stec, 2006). A major feature of thionins is their wide range of toxic effects on different biological systems. Most studied thionins are toxic to bacteria, fungi, yeasts and various mammalian cell types *in vitro*. The toxic effects seem to be based on the destruction of the organization of cell membranes by the interaction with negatively charged phospholipids. This causes a disturbance of the Ca²⁺ or K⁺ balance, and results in the leakage of proteins, nucleotides and other components (Bohlmann and Apel, 1991; Thevissen *et al.*, 1996). In addition to the damage of pathogen membranes, García-Olmedo *et al.* (1983) demonstrated an inhibitory effect of thionins on microbial protein synthesis. Thionin genes are not only induced by pathogen attack in plants, but are also regulated by chemical inducers, such as heavy metals (Fischer *et al.*, 1989), 2,6-dichloroisonicotinic acid (Wasternack *et al.*, 1994) and plant hormones, such as jasmonic acid or methyl jasmonate (MeJA) (Andresen *et al.*, 1992; Epple *et al.*, 1995; Kitanaga *et al.*, 2006; Vignutelli *et al.*, 1998), salicylic acid (SA) (Kogel *et al.*, 1995), gibberellic acid and brassinolide (Kitanaga *et al.*, 2006). Further support for a role of thionins as defence peptides comes from studies in which exogenous or endogenous thionin genes are overexpressed in plants. Overexpression of plant thionin genes leads to enhanced resistance against a broad range of bacterial and fungal pathogens (Bohlmann and Broekaert, 1994; Chan *et al.*, 2005; Epple *et al.*, 1997; García-Olmedo *et al.*, 1998; Holtorf *et al.*, 1998; Iwai *et al.*, 2002; Muramoto *et al.*, 2012; Shirasawa-Seo *et al.*, 2002).

Although plants possess protective mechanisms against their pathogens, their defensive network is not infallible. To successfully infect a plant, pathogens have evolved virulence molecules. For example, effectors secreted from pathogens can overcome, manipulate or suppress host defence and help to establish compatible interactions (Nomura *et al.*, 2005; Van Loon *et al.*, 2006). *Oryza sativa* roots are susceptible to a variety of pathogens, among which are plant-parasitic nematodes and oomycetes. In recent years, rice production has shifted from traditional paddy

*Correspondence: Email: Tina.Kyndt@ugent.be

systems to water-saving systems, such as aerobic rice production. However, successive aerobic rice cropping has been observed to result in a progressive decline in yield (Peng *et al.*, 2006; Ventura and Watanabe, 1978). These declining yields are associated with nutrient deficiencies (Kreye *et al.*, 2009; Nie *et al.*, 2008), as well as biotic factors, in which root-affecting *Pythium* spp. (*Pythium graminicola* and *Pythium arrhenomanes*) and root-knot nematodes (RKNs), such as *Meloidogyne graminicola*, are the predominant species (Bridge *et al.*, 2005; Dangal *et al.*, 2009; De Waele and Elsen, 2007; Soriano *et al.*, 2000; Tandingan *et al.*, 1996; Van Buyten *et al.*, 2013). In the case of RKNs, infective second-stage juveniles (J2) penetrate the roots and migrate intercellularly towards the root apex, where they make a U-turn and move upwards in the vascular bundles to the differentiation zone, where they settle and initiate permanent feeding sites called giant cells. These large, multinucleated, metabolically active giant cells are the nutrient source from which the nematode feeds for the remainder of its sedentary life (Gheysen and Mitchum, 2011; Kyndt *et al.*, 2013). As a result of hyperplasy and hypertrophy of the surrounding cells, galls appear, typically hook-formed and terminal in the case of *M. graminicola*.

After rapid colonization of rice roots and the rice seed surface, *Pythium* spp. may kill the seed embryo prior to germination or penetrate seedling roots directly and spread intra- and intercellularly as hemi-biotrophic pathogens (Chun and Schneider, 1998; De Vleeschauwer *et al.*, 2012; Van Buyten and Höfte, 2013). Strong root necrosis and nutrient depletion stimulate the end of the pathogen life cycle, which is paralleled by oospore production inside the tissue.

A previous transcriptome study of galls caused by *M. graminicola* infection at 3 and 7 days post-inoculation (dpi) in rice revealed that, instead of up-regulation, as expected for PR proteins on pathogen infection, most rice thionin genes on chromosome 6 were down-regulated (Kyndt *et al.*, 2012a). Moreover, thionin genes on chromosome 6 were also attenuated in roots at 2 dpi after *P. graminicola* infection [data available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database under the reference GSE32582]. This may be the consequence of *M. graminicola*- or *P. graminicola*-mediated suppression of plant defence, indicating that rice thionins on chromosome 6 may be resistance factors against *M. graminicola* and *P. graminicola*. Fourteen of a total of at least 39 annotated rice thionin genes are located on chromosome 6, and their coding sequences and amino acid sequences are highly similar (Ouyang *et al.*, 2007). It is probable that these thionin genes on chromosome 6 have evolved through gene duplication, a very important process for living organisms to acquire novel genes and to defend themselves against rapidly evolving pathogens (Martin and McInerney, 2009). To obtain more insight into the functions of rice thionins, we selected three rice thionin genes from rice chromosome 6 for further study, based on their strongly down-regulated

expression pattern inside infected tissues (Kyndt *et al.*, 2012a, GSE32582). We first confirmed and extended a time course of the expression pattern of these three selected rice thionin genes in different developmental stages of galls, using quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) analyses and promoter- β -glucuronidase (promoter-*GUS*) studies. Subsequently, *OsTH17* was overexpressed in *O. sativa* cv. Nipponbare to evaluate its protective effect against *M. graminicola* and *P. graminicola* infection. Finally, the regulation of the three selected rice thionin genes in response to plant defence-related hormones was studied by qRT-PCR and promoter-*GUS* analyses.

RESULTS

Rice thionin genes show lower expression in *M. graminicola*-induced galls than in corresponding non-inoculated root tips

In order to analyse the expression of rice thionin genes in more detail during the compatible interaction between *M. graminicola* and *O. sativa*, the expression of different rice thionin genes from chromosome 6 was investigated by qRT-PCR on galls and corresponding non-inoculated root tips as controls (Fig. 1). As a result

Fig. 1 Relative expression levels based on quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) of three rice thionin genes in *Meloidogyne graminicola*-induced galls compared with non-inoculated root tips at different time points after inoculation (dpi). Gene expression levels were normalized using three internal reference genes, *OsEXP*, *OsUBQ5* and *OsEIF5C*. Bars represent the mean expression level $\pm$ SE from two independent biological and three technical replicates, each containing a pool of eight plants. Data were analysed using REST2009 software. The black horizontal line shows the expression level in the non-inoculated control plants (set at 1). Significant differential expression ($P \leq 0.05$) in comparison with non-inoculated root tips (control expression level = 1) is indicated with an asterisk.

Fig. 2 Spatio-temporal localization of β -glucuronidase (GUS) in *Meloidogyne graminicola*-induced galls and in the root of a non-inoculated *pOsTHI7::GUS* line: (a) 2 days post-inoculation (dpi); (b) 3 dpi; (c) 7 dpi; (d) 14 dpi; (e, f) non-inoculated root tips corresponding to the 7- and 14-dpi time points. Two independent *pOsTHI7::GUS* lines were used in this study, with similar results. Scale bar, 200 μ m.

of the high similarity, it was impossible to design specific primer pairs for *OsTHI7* and *OsTHI6* to be used in SYBR-Green qRT-PCR; hence, the results from this primer pair are described as *OsTHI7* + 6. The gene expression analysis was performed at four time points representing the whole life cycle of *M. graminicola*. All analysed thionin genes showed significantly lower expression in galls than in control root tips until 7 dpi, when giant cells matured. Even at 14 dpi, *OsTHI7* + 6 still showed lower expression in galls than in control root tips, although this difference was not significant. However, *OsTHI1* and *OsTHI2* were strongly induced at 14 dpi.

These qRT-PCR data confirm and extend the transcriptome analysis of *M. graminicola*-induced galls (Kyndt *et al.*, 2012a; Table S1, see Supporting Information). Based on its consistent down-regulation in galls, *OsTHI7* was used as a candidate for further study. First, 14 independent *OsTHI7*-promoter::*GUS* lines were generated, and two of these lines were used to analyse the spatial and temporal localization of GUS inside galls. In non-inoculated rice seedlings, GUS was only localized in the root tips, indicating that *pOsTHI7* was active at the distal end of rice roots (Fig. 2e,f). The spatio-temporal localization of GUS in *M. graminicola*-induced galls was also analysed in these lines. GUS intensity decreased gradually during the development of galls (Fig. 2a–d). We further investigated GUS expression in giant cells by cross-sectioning 7-dpi galls and corresponding root tips. As shown in Fig. 3, no GUS activity (pink staining) was observed in 7-dpi giant cells, whereas pink staining was clearly seen in the epidermis, cortex and vascular cells of the non-inoculated root tip sections. In conclusion, qRT-PCR data and histochemical analysis

of GUS activity in *pOsTHI7::GUS* lines shows that, under normal growth conditions, *OsTHI7* exhibits a root tip-specific expression pattern, and its expression is lower in galls than in non-infected root tips. *OsTHI7* promoter activity is not detectable inside 7-dpi giant cells induced by *M. graminicola*.

Overexpression of *OsTHI7* in rice decreases susceptibility to *M. graminicola*

To determine whether *OsTHI7* can protect rice against *M. graminicola* infection, 30 independent T0 *OsTHI7*-overexpressing lines were generated. All the transgenic lines looked phenotypically normal. The transformation was validated by PCR and semi-quantitative RT-PCR (data not shown). The same vector, without insert, was also transformed to generate control plants (empty vector line).

Three T1 hemizygous *OsTHI7*-overexpressing lines with a single insertion (tested by segregation analysis) and high expression (Fig. S1a, see Supporting Information) were used in an infection experiment. The overexpressing lines possessed *OsTHI7* + 6 expression levels approximately 8200-fold higher than in empty vector or wild-type controls, although the calculation of the exact levels in the controls may be hindered because of the very low basal level. This phenomenon has also been observed by others (Oñate-Sánchez *et al.*, 2007). In a semi-quantitative RT-PCR analysis, after 25 cycles, *OsTHI7* + 6 expression was not detectable in wild-type or empty vector roots, whereas its expression level was already very high in the transgenic overexpressing lines. It has been demonstrated previously that transgene expression or

Fig. 3 Thin section of a gall at 7 days post-inoculation (dpi) and corresponding root tip. β -Glucuronidase (GUS) staining (pink colour) was visualized by dark field microscopy: (a) 7-dpi gall section; (b) root tip section. *Giant cell; scale bar, 100 μ m.

epigenetic effects caused by the transformation process can disrupt the homeostasis of plant cells and lead to the expression of defence-related proteins (Epple *et al.*, 1997; Herbers *et al.*, 1996; Mittler *et al.*, 1995). However, in the current study, the expression of *PR1a* and *PR1b* was not changed in the *OsTHI7*-overexpressing plants compared with the empty vector or wild-type controls (Fig. S1b).

Two weeks after inoculation with *M. graminicola*, root and shoot length, number of galls and different stages of nematodes were evaluated. At this time point, the root length of these five lines was not significantly different (Fig. S2, see Supporting Information); however, the shoot length of two *OsTHI7*-overexpressing lines (OX19 and OX22) was significantly greater than that of the control plants, and the shoot length of the third *OsTHI7*-overexpressing line (OX2) was significantly greater than that of wild-type plants, but not of empty vector plants (Fig. S2). The number of galls was not significantly different in the *OsTHI7*-overexpressing lines compared with control plants (Fig. 4a). However, there was a significant reduction in the number of females and total nematodes per plant (average of 39.2% reduction, Fig. 4b) in the overexpressing plants in comparison with both control lines.

Overexpression of *OsTHI7* reduces susceptibility of rice to *P. graminicola*

As *OsTHI7* was also attenuated in roots after virulent oomycete *P. graminicola* infection, as shown by a microarray study (Table S1, GSE32582), we tested the susceptibility of one overexpressing line (OX22) to *P. graminicola* infection. Seven days after *P. graminicola* infection, roots of wild-type, empty vector and *OsTHI7*-overexpressing plants all developed typical brown necrotic patches. There were no significant differences in disease rates of the root systems of control and overexpressing plants (Fig. 5a). However, shoots of the OX22 line appeared significantly healthier than those of the control lines, with a lower disease rate and a higher shoot length (Fig. 5b–d). To quantify *Pythium* colo-

nization in the roots, qPCR was conducted on the DNA of surface-sterilized roots. *Pythium* DNA was quantified using the method described by Van Buyten *et al.* (2013), and plant DNA was quantified using a rice housekeeping gene (LOC_Os07g02340). The data indicated that *P. graminicola* massively infects the whole root system in wild-type and empty vector lines, where *P. graminicola* DNA quantities represent 97% and 98%, respectively, of the total DNA extract at 7 dpi. However, the quantity of *P. graminicola* DNA was much lower in the *OsTHI7* line, representing 70% of the total DNA extract (Fig. 6a).

In order to further investigate the colonization of *P. graminicola* in the roots of the different rice lines (wild-type, empty vector and *OsTHI7*-OX22), root sections were examined under bright field microscopy after trypan blue staining (Fig. 6b). At 7 dpi, the infected parts of the primary roots of wild-type and vector lines were strongly colonized by *P. graminicola*. The oomycete heavily colonized the entire root system, with the hyphae spreading extensively from the epidermis through the cortex to the vascular bundle. However, in the *OsTHI7*-OX22 line, *P. graminicola* was mainly constricted to the root epidermis, whereas the cortex and vascular bundle appeared to be invaded only slightly.

Rice thionin genes respond differentially to plant hormones

Previous studies have shown that many PR genes, including plant thionin genes, respond differentially to phytohormones (Bohlmann *et al.*, 1998; Iwai *et al.*, 2002; Kitanaga *et al.*, 2006; Sels *et al.*, 2008; Yazaki *et al.*, 2003). Initial predictions using PLANT CARE (Lescot *et al.*, 2002) indicated several hormone-related cis-elements in the promoter region of rice thionin genes (data not shown). Therefore, the expression pattern of the three selected thionin genes was further investigated by qRT-PCR in plants treated with hormones (or their analogues).

Figure 7 shows that the root expression of the three thionin genes responds differentially to plant hormone (or analogue) application. Six and 24 h after abscisic acid (ABA) treatment, the

Fig. 4 *OsTH17*-overexpressing plants exhibit decreased susceptibility to *Meloidogyne graminicola*. Data obtained 14 days after inoculation (dpi). (a) The number of galls in overexpressing (OX2, OX19 and OX22) and control (WT and empty vector) plants. (b) The different developmental stages of *M. graminicola* within the galls in overexpressing and control plants. Bars represent the mean $\pm$ SE of eight plants. Different letters indicate statistically significant differences (Duncan's multiple range test with $P \leq 0.05$). a/b indicate significant differences in second-stage juveniles (J2s); A/B indicate significant differences in J3 + J4 stages. α/β indicate significant differences in females; θ/ϵ indicate significant differences in the total number of infected nematodes. Data represent one of three independent experiments with similar results. WT, wild-type; Vec, transgenic line containing an empty vector; OX, *OsTH17*-overexpressing line.

three thionin genes were significantly up-regulated; *OsTH1* showed consistent induction at all tested time points (Fig. 7a). These results indicate that these thionin genes could be involved in the ABA-mediated defence pathway. *OsTH1* was responsive to MeJA at all time points (Fig. 7b); *OsTH2* and *OsTH7 + 6* were up-regulated after 6 h of treatment, but then attenuated gradually. After 72 h of treatment, the mRNA levels of both genes were significantly down-regulated, which could be the result of feedback effects and/or modulation by cross-talk with other plant hormones. On treatment with an SA analogue, benzo-1,2,3-thiadiazole carbothioic acid *S*-methyl ester (BTH) (Fig. 7c), *OsTH7 + 6* was persistently induced at all the investigated time points, whereas *OsTH1* showed a very minor induction after 6 h of BTH treatment. *OsTH2* was not altered significantly by this treatment.

For the validation of qRT-PCR data, GUS activity was studied in a *pOsTH7::GUS* line 6 h and 3 days after phytohormone (or analogue) treatment. GUS assays revealed similar responses to hormone (or analogue) treatment as in qRT-PCR (Fig. S3, see Supporting Information).

DISCUSSION

Thionins, identified as antimicrobial peptides and PR proteins, have been studied for decades (Bohlmann and Apel, 1991; Edreva, 2005). Although there are a large number of thionin genes in the rice genome, no *in vivo* or *in vitro* research has focused on the functional analysis of rice thionins.

In this study, the role of rice thionin genes in defence against the RKN *M. graminicola* and the oomycete *P. graminicola* was investigated. The expression of thionin genes in root galls caused by *M. graminicola* was studied using qRT-PCR. Four time points

were chosen for this analysis: 2 and 3 dpi are the time points at which *M. graminicola* starts to establish a feeding site; at 7 dpi, the giant cells are fully mature; and, at 14 dpi, the females start to produce eggs. *OsTH1* and *OsTH2* were both strongly down-regulated in 2-, 3- and 7-dpi galls, but considerably up-regulated at 14 dpi. This is probably a result of the damage caused by the massive swelling of the root system induced by the growing nematodes and the egg masses produced. *OsTH7 + 6* was expressed to a lesser extent in galls than in corresponding non-inoculated root tips, at all evaluated time points, although, at 14 dpi, the difference was not significant. Down-regulated thionin genes were also observed in *M. incognita*-infected rice roots at 6 dpi (GSE21919) and in *M. graminicola*-infected rice roots at 4 dpi (GSE21919), as well as in other biotic stress conditions (GSE32426, GSE10373). Down-regulation of thionin genes was reported in the compatible interaction of barley with a virulent strain of *Erysiphe* (= *Blumeria*) *graminis* f. sp. *hordei* compared with non-infected leaves. Nevertheless, in an incompatible interaction, the level of thionin expression was not changed or slightly enhanced compared with control leaves (Ebrahim-Nesbat *et al.*, 1993). These studies could indicate that a lower expression of thionin genes somehow contributes to successful infestation. As *OsTH7 + 6* was consistently down-regulated, we decided to analyse the effect of *OsTH7* overexpression for further elucidation of the role of *OsTH7* in rice defence against *M. graminicola*. The study of GUS activity in *pOsTH7::GUS* plants showed that, under non-stressed conditions, *OsTH7* was weakly expressed at the root tip. This peptide may have a function to protect the fragile root tips from certain soil pathogens or insect infestation. Contrary to our observations, Iwai *et al.* (2002) detected no thionin gene expression at all in 2-week-old rice roots, although they found the expression of endogenous thionin genes (several homologous genes) in coleoptiles of 4-day-

Fig. 5 *OsTHI7*-overexpressing line (OX22) reduced the susceptibility of rice to *Pythium graminicola* infection compared with control (WT and empty vector) lines. Data obtained at 7 days post-infection. (a) Disease rating (0–V) on roots of control and *OsTHI7*-overexpressing plants. (b) Disease rating (0–V) on shoots of control and *OsTHI7*-overexpressing plants. (c) Average shoot length per plant in the different lines, either *P. graminicola* or mock infected. (d) Representative photographs taken at the time of disease scoring. Statistical analysis for disease rating was performed using the Mann–Whitney test ($P \leq 0.05$). Statistical analysis for shoot length was performed using Duncan's multiple range test ($P \leq 0.05$). Different letters indicate statistically significant differences. Bars represent the mean $\pm$ SE of 12 plants. Data represent one of three independent experiments with similar results. WT, wild type; Vec, transgenic line containing an empty vector; OX22, *OsTHI7*-overexpressing line 22.

old plants using Northern blot. This difference may be a result of the different methodologies or materials used.

The toxicity of PR proteins is generally dose dependent (Stec, 2006) and, knowing that the cultivar Nipponbare is highly susceptible to *M. graminicola*, we might conclude that the endogenous *OsTHI7* level in the root tip is not sufficiently high to protect the roots from *M. graminicola* infection. Nevertheless, our data

showed that, as a result of an as yet unknown mechanism, thionin gene expression was much weaker in galls than in non-inoculated root tips, and this may prevent the peptide from reaching the toxic dose for *M. graminicola*. Whether this low expression is caused by active suppression of the immune system by nematode effectors remains to be elucidated. Our qRT-PCR results were confirmed by the *pOsTHI7::GUS* assays, where we also observed that there was

Fig. 6 *OstH17*-overexpressing line (OX22) is colonized to a lesser extent than control (WT and empty vector) lines by *Pythium graminicola*. (a) *In planta* quantification of *P. graminicola* at 7 days post-inoculation (dpi) in different lines using quantitative polymerase chain reaction (qPCR). qPCR was conducted on infected and surface-sterilized root DNA samples. Pure *P. graminicola* DNA and non-infected plant DNA were used to make standard curves. LOC_Os07g02340 was used for plant DNA normalization. Bars represent the mean percentage of *P. graminicola* DNA in total extract DNA $\pm$ SD from three technical replicates and two biological replicates, each containing a pool of six plants. WT, wild type; Vec, transgenic line containing an empty vector; OX, *OstH17*-overexpressing line. Different letters indicate statistically significant differences (Duncan's multiple range test with $P \leq 0.05$). (b) Colonization of *P. graminicola* in the most heavily infected parts of primary roots at 7 dpi. Cross-sections (10 μ m) from *P. graminicola*-infected root tissues were stained with trypan blue. Scale bars, 200 μ m. Control, wild-type + mock; WT, wild-type + *P. graminicola*; Vec, transgenic line containing an empty vector + *P. graminicola*; OX22, *OstH17*-overexpressing line 22 + *P. graminicola*.

no GUS activity inside 7-dpi giant cells and their neighbouring cells, whereas this gene was expressed throughout the epidermis, cortex and vascular tissue of non-inoculated root tip sections. This result is also consistent with our study on giant cell transcripts by mRNA-Seq, which showed that *OstH17* was not expressed inside giant cells (Ji *et al.*, 2013).

Next, we overexpressed *OstH17* in the susceptible cultivar Nipponbare. The three tested *OstH17*-overexpressing lines showed less severe infection by *M. graminicola*. The enhanced resistance of the transgenic lines is probably a result of a direct toxic effect of OSTH17 on *M. graminicola*. Although we have not shown that OSTH17 has a toxic effect on nematodes, this peptide has all the characteristics important for toxicity (Bohlmann and Apel, 1991; Stec, 2006). It is basic and contains tyrosine-13 (Tyr13) and lysine-1 (Lys1), two residues that determine toxicity by altering the permeability of cell membranes in various organisms, such as in cultured mammalian cells or fungal cells (Stec, 2006). Colgrave *et al.* (2010) reported that cyclotides, small disulfide-rich proteins having antimicrobial activity and insecticidal activity via disruption of cell membranes, had a toxic effect on the gastrointestinal nematode *Haemonchus contortus* of sheep, acting through its epicuticle. Nevertheless, the exact mode of action of OSTH17 against *M. graminicola* needs to be studied in more detail.

When the roots of *OstH17*-OX22 were infected with *P. graminicola*, a root oomycete, the plants showed a significantly lower shoot disease score compared with control plants. Although root necrosis was still observed in the *OstH17*-OX22 line, qPCR results confirmed that there was less *Pythium* colonization in its roots than in control roots. Histological studies revealed that *P. graminicola* massively parasitized the whole root in wild-type and empty vector lines, as observed by dense hyphal networks in the epidermis, cortical cells and vascular bundles. The abundant hyphae in vascular tissue may block water and nutrient transport to shoots, resulting in wilt and seedling death after *P. graminicola* infection (Van Buyten *et al.*, 2013). In the case of the *OstH17*-OX22 line, the hyphae were largely restricted to the epidermis, giving rise to the observed necrosis, and occasionally occurring in a few cortical and vascular cells. This colonization pattern explains the observation that there was almost no wilting of shoots in the infected *OstH17*-OX22 line. Whether the lower colonization by *P. graminicola* is caused by a toxic effect of OSTH17 still needs to be explored.

Phytohormones not only coordinate all aspects of plant growth and development, but also modulate numerous stress- and defence-related responses in plants, such as the expression of PR proteins (Agrawal *et al.*, 2000; Pieterse *et al.*, 2009, 2012). To understand the regulation of rice thionin genes by phytohormones, the accumulation of the three selected thionin transcripts in rice roots was assessed after BTH, MeJA and ABA treatment of plant roots. The results revealed that all the transcripts were up-regulated by the three molecules at 6 h, the ear-

Fig. 7 Analysis of the expression level of thionin genes in rice roots after root soaking in 50 μ M abscisic acid (ABA) (a), 100 μ M methyl jasmonate (MeJA) (b) and 250 μ M benzo-1,2,3-thiadiazole carbothioic acid *S*-methyl ester (BTH) (c) for 6 h, 24 h and 3 days. Gene expression levels were normalized using three internal reference genes: *OsEXP*, *OsUBQ5* and *OsEIF5C*. Bars represent the mean expression level $\pm$ SE from two independent biological and three technical replicates, each containing a pool of eight plant roots. Data were obtained using REST2009 software. Significant differential expression ($P \leq 0.05$) is indicated with an asterisk in comparison with non-treated control roots (expression level = 1). The black horizontal line shows the expression level in the control plants (set at 1).

liest time point in our test. The induction of the three rice thionin genes by treatment with BTH (an SA analogue), MeJA and ABA at 6 h indicates that these thionin genes may function as early defence genes. Our results demonstrated that *OsTHI1*, *OsTHI2* and *OsTHI7 + 6* were involved in the ABA-mediated stress response, as these thionin genes responded strongly to ABA treatment. The presence of the ABRE motif in the promoter regions of the thionin genes studied here suggests that they may be regulated directly by ABA. ABA has been shown to play an important role in plant responses to abiotic and biotic stress (Chinnusamy *et al.*, 2008; Cramer *et al.*, 2011; Wan and Li, 2006). Yazaki *et al.* (2003) reported that *OsTHI2* was up-regulated in 30-day-old rice calli after a 3-day treatment with either 50 μ M ABA or 50 μ M gibberellic acid. It has been reported that the exogenous application of MeJA to rice leaves significantly enhances defence against *M. graminicola* in roots (Nahar *et al.*, 2011). The consistent induction of *OsTHI1* and the short-term effect on *OsTHI2* and

OsTHI7 + 6 after MeJA treatment might contribute in part to the MeJA-induced resistance against *M. graminicola*. When plants were treated with BTH, *OsTHI7 + 6* was induced dramatically, and Nahar *et al.* (2011) reported that, although less strong than MeJA and ethylene, the exogenous application of BTH induced systemic defence in rice roots to *M. graminicola*. Taken together with our infection experiments, these results indicate that *OsTHI7* may participate in MeJA- and SA-mediated resistance against *M. graminicola*. Although in our qRT-PCR analyses, *OsTHI7* and *OsTHI6* could not be distinguished, the qRT-PCR-derived expression pattern of *OsTHI7 + 6* was consistent with the expression of *GUS* in the *pOsTHI7::GUS* line, which indicates that *OsTHI7* and *OsTHI6* are regulated in a similar way. Moreover, there is high similarity in the promoter sequences of *OsTHI7* and *OsTHI6* (98% identity). The differential expression pattern of the three thionin genes on chemical treatments indicates that, although there are strong similarities in amino acid sequences among the thionin

peptides, the response of the thionins to different stresses may be different and diverse, as a consequence of evolution.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Seeds of *O. sativa* cv. Nipponbare (GSOR-100; Genetic Stocks Oryza Collection, Washington DC, USA) were germinated on wet filter paper for 3 days at 30 °C and then transferred to SAP (sand + absorbent polymer) substrate (Reversat *et al.*, 1999) in polyvinylchloride (PVC) tubes and grown at 26 °C under a 16-h/8-h light regime at a relative humidity of 70%–75%.

Gene isolation, vector construction and rice transformation

The *OsTH17* genomic DNA sequence including two introns (737 bp) and the DNA sequence upstream of the *OsTH17* start codon (1994 bp) were amplified separately using PCR on DNA of *O. sativa* cv. Nipponbare. The primer sequences are provided in Table S2 (see Supporting Information). The vector construction was performed by Gateway® cloning (Life Tech, Merelbeke, Belgium). Vector pMBb7Fm21GW-UBIL was employed for *OsTH17* overexpression using the maize ubiquitin promoter, and pBGWFS7.0 was used for promoter-*GUS* line construction. Both vectors were obtained from Plant Systems Biology [Vlaams Instituut voor Biotechnologie (VIB), Ghent, Belgium]. All the inserted fragments were sequenced before introduction into *Agrobacterium*.

The binary vectors were introduced into *Agrobacterium tumefaciens* EHA105 cells using triparental mating. Transformed cells were selected on YEB agar plates (5 g/L beef extract, 5 g/L peptone, 1 g/L yeast extract, 5 g/L sucrose) containing spectinomycin (100 µg/mL). Rice transformation was performed according to an in-house protocol based on the protocols of Zhang *et al.* (1997), Hiei *et al.* (1994) and Paine *et al.* (2005). Transformed calli were selected using 50 µg/mL glufosinate (Sigma-Aldrich, Diegem, Belgium), and the regenerated rice plants were grown in a growth chamber under 16-h/8-h of light/dark, 26 °C and 70%–75% relative humidity. As a control, transgenic lines containing the pMBb7Fm21GW-UBIL vector without insert (empty vector) were generated.

Segregation analysis was performed on 70 T1 seedlings by selection on 8 mg/L glufosinate, and the results were analysed with the χ^2 test ($P = 0.05$). Three overexpressing lines and two promoter-*GUS* lines with a 3:1 segregation were used for further experiments. One line transformed with the empty vector and *O. sativa* cv. Nipponbare (wild-type) were used as controls.

Nematode infection assay

The *M. graminicola* culture was provided by Professor Dirk De Waele (Catholic University, Leuven, Belgium) and was originally isolated in the Philippines. *Meloidogyne graminicola* was maintained on *O. sativa* cv. Nipponbare in potting soil under the same temperature/light conditions as described above, and the nematodes were extracted using a modified Baermann funnel. Fourteen-day-old rice plants were inoculated with about 250 J2s of *M. graminicola* per plant or mock inoculated with water. To test

thionin expression by qRT-PCR, 2-, 3-, 7- and 14-dpi galls and corresponding non-inoculated root tips were collected and stored at –80 °C for RNA extraction. For the infection experiment on transgenic plants, the infection level of the plants was evaluated at 14 dpi by counting the number of galls and nematodes per plant. To visualize the galls and nematode developmental stages, roots were stained with acid fuchsin, as described by Nahar *et al.* (2011). Each experiment included at least eight individual plants per treatment. The whole experiment was repeated three times independently in space and time.

qRT-PCR analyses

RNA extraction and cDNA synthesis were performed and analysed as described by Kyndt *et al.* (2012b). qRT-PCR was performed and analysed as described by Nahar *et al.* (2011) on T1 generation roots. The primer sequences used for qRT-PCR are listed in Table S2. In all qRT-PCR experiments, the expression of both *OsTH17* and *OsTH16* was detected simultaneously, as these two transcript sequences (including the untranslated region) are highly similar, and hence it was impossible to design gene-specific primers for SYBR-Green-based qRT-PCR. All qRT-PCRs were performed in triplicate on cDNA from two independent biological replicates, each containing a pool of eight plants.

Histological analysis of *OsTH17* promoter::*GUS* activity

Histochemical *GUS* activity of whole seedlings was investigated as described by Delporte *et al.* (2011) with a few modifications. After overnight reaction in X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) solution, the samples were washed several times using 70% ethanol, and stored in 100% ethanol for a few days to wash out the excess dye and chlorophyll. Before transfer to microscopic slides, seedlings were incubated in lactic acid to clear the plant materials; *GUS* staining was observed under a binocular microscope (Leica S8APO, Leica Microsystems, Nussloch, Germany).

To observe *GUS* in giant cells, *GUS*-stained galls and control root tips were first rehydrated in 50% ethanol for 2 h and 30% ethanol for 2 h, and then incubated in water overnight. The materials were then fixed in 50 mM sodium phosphate buffer (pH 7.2) containing 4% paraformaldehyde and 1% glutaraldehyde overnight, and subsequently dehydrated in a graded ethanol series (15%, 30%, 50%), each for 2 h, and then dehydrated in 70% ethanol overnight. The next day, the samples were further dehydrated in 85% and 100% ethanol, respectively, for 2 h. Afterwards, the samples were incubated in 100% ethanol overnight. The samples were infiltrated with 50% Technovit 7100 + 50% ethanol solution (Heraeus Kulzer, Wehrheim, Germany) overnight at 4 °C, after which the infiltration solution was replaced by 100% Technovit 7100 plus Hardner I and the samples were stored overnight at 4 °C. The infiltrated materials were embedded in plastic cubes filled with Technovit 7100 plus Hardner II and kept at 37 °C overnight. Embedded samples were sectioned at 10 µm in a Leica RM2265 motorized rotary microtome (Leica Microsystems), and *GUS* staining was observed in a dark field using a binocular microscope (Leica S8APO, Leica Microsystems).

Pythium graminicola bioassay

Pythium graminicola strain PB912 132 culture, inoculation and disease monitoring were performed according to De Vleeschauwer *et al.* (2012)

and Van Buyten and Höfte (2013). *Pythium graminicola* strain PB912 132 (Van Buyten and Höfte, 2013), isolated from a diseased aerobic rice field in Los Banos, Philippines, was cultivated on potato dextrose agar (PDA; Difco Laboratories, Bierbeek, Belgium) at 28 °C. The husks of rice seeds (wild-type, empty vector and OX22) were removed and the seed surfaces were sterilized with 6% sodium hypochlorite for 20 min, and subsequently rinsed three times with sterile water. Sterilized seeds were grown in square Petri dishes (12 × 12 cm²) on standard-strength Gamborg B5/1% plant agar medium at 28 °C (day)/26 °C (night) under a 12-h photoperiod. Four days after germination, seedlings were inoculated with PDA plugs containing 4-day-old *P. graminicola* strain PB912 132 in between two plants. Control samples were mock inoculated with the same size of PDA plugs. Disease symptoms were recorded at 7 dpi. Disease symptoms on rice shoots were rated on the basis of a disease severity scale according to Van Buyten and Höfte (2013), and disease symptoms on rice roots were rated on the basis of a disease severity scale according to De Vleeschauwer *et al.* (2012). For each line, 12 seedlings were analysed in two Petri dishes per experiment. The experiment was independently repeated three times with similar results.

Microscopic analysis of root samples infected with *P. graminicola*

Infected root samples (7 dpi), ± 0.5 cm in length, were collected, and the fixation, dehydration and embedding procedures were the same as described above for GUS observation in giant cells and root tips. Sections (10 µm) were produced using a Leica RM2265 rotary microtome; from each line, in total about 30 sections were produced from six individual seedlings. *Pythium graminicola* hyphae were stained by incubating sections in 0.1% (w/v) trypan blue in 10% (v/v) acetic acid for 5 min. Images were visualized with an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with an Olympus ColorView III camera and xenon light source. Images were processed with Olympus analysis cell[®]F software (Olympus Soft Imaging Solutions, Munster, Germany). About eight randomly chosen sections were analysed for each line, all showing the same trend.

Quantitative detection of *P. graminicola* in rice roots

Pythium graminicola-infected roots were collected at 7 dpi, surface sterilized in 1% NaOCl for 1 min and then thoroughly washed. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands). The quality and concentration of the extracted DNA were determined with a Nanodrop 2000 spectrophotometer (Thermoscientific, Aalst, Belgium). qPCR was performed in triplicate on DNA from two independent biological replicates, each containing a pool of six infected plant roots. Pure *P. graminicola* DNA and non-infected plant DNA were used to make standard curves. Primers specific for the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) of *P. graminicola* PB912 132 and primers for plant DNA (reference gene LOC_Os07g02340) are shown in Table S2.

Chemical treatment

Five-day-old seedlings were grown hydroponically in 50% Hoagland solution. After 5 days, Hoagland solution was supplemented with MeJA

(100 µM, Sigma-Aldrich NV/SA, Bornem, Belgium), ABA (50 µM, Sigma-Aldrich) or BTH (250 µM, Syngenta Crop Protection, Brussels, Belgium) solutions, which were prepared in 50% Hoagland solution, and pure 50% Hoagland solution was used as a control. The concentrations used have been shown to induce root defence responses in rice against RKNs (Nahar *et al.*, 2011, 2012). After transfer to the hormone (or analogue) solution, root samples were collected at 6, 24 and 72 h for RNA extraction to study thionin gene expression. Two biological replicates, each composed of a pool of eight individual plants, were taken.

Data analysis

Data obtained from qRT-PCR were analysed using the software REST2009 (Qiagen). All other statistical analyses were performed using the software SPSS (version 21, IBM, USA). The normality of the data was checked using the Kolmogorov–Smirnov test of composite normality ($\alpha = 0.05$). Homogeneity of variance was checked by the Levene test ($P \leq 0.05$). Analysis of variance (one-way ANOVA) and multiple comparisons of differences between treatments were also performed (Duncan's multiple range test, $P \leq 0.05$). The *Pythium* disease rate was analysed by the non-parametric Mann–Whitney test ($P \leq 0.05$). GSE numbers were obtained from Genevestigator (Hruz *et al.*, 2008).

ACKNOWLEDGEMENTS

We acknowledge the financial support of Geconcerteerde OnderzoeksActie UGent (GOA) 01GB3013 and Fonds Wetenschappelijk Onderzoek-Vlaanderen (FWO) G.0315.13. We thank Lien De Smet for excellent technical assistance. H.J. was supported by a BOF PhD scholarship (Bijzonder Onderzoeksfonds, Ghent University). T.K. was supported by an FWO postdoctoral fellowship.

REFERENCES

- Agrawal, G.K., Jwa, N.S. and Rakwal, R. (2000) A novel rice (*Oryza sativa* L.) acidic PR1 gene highly responsive to cut, phytohormones, and protein phosphatase inhibitors. *Biochem. Biophys. Res. Commun.* **274**, 157–165.
- Andresen, I., Becker, W., Schlüter, K., Burges, J., Parthier, B. and Apel, K. (1992) The identification of leaf thionin as one of the main jasmonate-induced proteins of barley (*Hordeum vulgare*). *Plant Mol. Biol.* **19**, 193–204.
- Bohlmann, H. and Apel, K. (1991) Thionins. *Annu. Rev. Plant Biol.* **42**, 227–240.
- Bohlmann, H. and Broekaert, W. (1994) The role of thionins in plant protection. *Crit. Rev. Plant Sci.* **13**, 1–16.
- Bohlmann, H., Vignutelli, A., Hilpert, B., Miersch, O., Wasternack, C. and Apel, K. (1998) Wounding and chemicals induce expression of the *Arabidopsis thaliana* gene *Thi2.1*, encoding a fungal defense thionin, via the octadecanoid pathway. *FEBS Lett.* **437**, 281–286.
- Bridge, J., Plowright, R.A. and Peng, D. (2005) Nematode parasites of rice. In: *Plant Parasitic Nematodes in Subtropical and Tropical Agriculture* (Luc, M., Sikora, R.M. and Bridge, J., eds), pp. 87–130. Wallingford, Oxfordshire: CABI Publishing.
- Chan, Y.L., Prasad, V., Chen, K.H., Liu, P.C., Chan, M.T. and Cheng, C.P. (2005) Transgenic tomato plants expressing an *Arabidopsis* thionin (*Thi2.1*) driven by fruit-inactive promoter battle against phytopathogenic attack. *Planta*, **221**, 386–393.
- Chen, X. and Ronald, P.C. (2011) Innate immunity in rice. *Trends Plant Sci.* **16**, 451–459.
- Chinnusamy, V., Gong, Z. and Zhu, J.K. (2008) Absciscic acid-mediated epigenetic processes in plant development and stress responses. *J. Integr. Plant Biol.* **50**, 1187–1195.
- Chun, S.C. and Schneider, R. (1998) Sites of infection by *Pythium* species in rice seedlings and effects of plant age and water depth on disease development. *Phytopathology*, **88**, 1255–1261.

- Colgrave, M.L., Huang, Y.H., Craik, D.J. and Kotze, A.C. (2010) Cyclotide interactions with the nematode external surface. *Antimicrob. Agents Chemother.* **54**, 2160–2166.
- Cramer, G.R., Urano, K., Delrot, S., Pezzotti, M. and Shinozaki, K. (2011) Effects of abiotic stress on plants: a systems biology perspective. *BMC Plant Biol.* **11**, 163.
- Dangal, N., Shrestha, S., Poudyal, D.S. and Adhikari, C. (2009) Infestation of rice root-knot nematode in rice nurseries in Chitwan. *Nepal J. Sci. Technol.* **10**, 45–49.
- Delporte, A., Lannoo, N., Vandenborre, G., Ongenaert, M. and Van Damme, E.J.M. (2011) Jasmonate response of the *Nicotiana tabacum* agglutinin promoter in *Arabidopsis thaliana*. *Plant Physiol. Biochem.* **49**, 843–851.
- De Vleeschauwer, D., Van Buyten, E., Satoh, K., Balidion, J., Mauleon, R., Choi, I.R., Vera-Cruz, C., Kikuchi, S. and Höfte, M. (2012) Brassinosteroids antagonize gibberellin- and salicylate-mediated root immunity in rice. *Plant Physiol.* **158**, 1833–1846.
- De Waele, D. and Elsen, A. (2007) Challenges in tropical plant nematology. *Annu. Rev. Phytopathol.* **45**, 457–485.
- Durrant, W. and Dong, X. (2004) Systemic acquired resistance. *Annu. Rev. Phytopathol.* **42**, 185–209.
- Ebrahim-Nesbat, F., Bohl, S., Heitefuss, R. and Apel, K. (1993) Thionin in cell walls and papillae of barley in compatible and incompatible interactions with *Erysiphe graminis* f. sp. *hordei*. *Physiol. Mol. Plant Pathol.* **43**, 343–352.
- Edreva, A. (2005) Pathogenesis-related proteins: research progress in the last 15 years. *Gen. Appl. Plant Physiol.* **31**, 105–124.
- Eppe, P., Apel, K. and Bohlmann, H. (1995) An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. *Plant Physiol.* **109**, 813–820.
- Eppe, P., Apel, K. and Bohlmann, H. (1997) Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. *Plant Cell*, **9**, 509–520.
- Fischer, R., Behnke, S. and Apel, K. (1989) The effect of chemical stress on the polypeptide composition of the intercellular fluid of barley leaves. *Planta*, **178**, 61–68.
- Fritig, B., Heitz, T. and Legrand, M. (1998) Antimicrobial proteins in induced plant defense. *Curr. Opin. Immunol.* **10**, 16–22.
- García-Olmedo, F., Carbonero, P., Hernández-Lucas, C., Paz-Ares, J., Ponz, F., Vicente, O., Vicente, O. and Sierra, J.M. (1983) Inhibition of eukaryotic cell-free protein synthesis by thionins from wheat endosperm. *Biochim. Biophys. Acta*, **740**, 52–56.
- García-Olmedo, F., Molina Fernández, A., Alamillo, J.M. and Rodríguez Palenzuela, P. (1998) Plant defence peptides. *Peptide Sci.* **47**, 479–491.
- Gheysen, G. and Mitchum, M.G. (2011) How nematodes manipulate plant development pathways for infection. *Curr. Opin. Plant Biol.* **14**, 415–421.
- Goodman, R. and Novacky, A. (1994) *The Hypersensitive Reaction in Plants to Pathogens: a Resistance Phenomenon*. Saint Paul, Minnesota, USA: APS Press.
- Herbers, K., Meuwly, P., Frommer, W.B., Metraux, J.P. and Sonnewald, U. (1996) Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway. *Plant Cell*, **8**, 793–803.
- Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.
- Holtorf, S., Ludwig-Müller, J., Apel, K. and Bohlmann, H. (1998) High-level expression of a viscotxin in *Arabidopsis thaliana* gives enhanced resistance against *Plasmodiophora brassicae*. *Plant Mol. Biol.* **36**, 673–680.
- Hruz, T., Laule, O., Szabo, G., Wessendorf, F., Bleuler, S., Oertle, L., Widnayer, P., Gruissem, W. and Zimmermann, P. (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformatics*, **2008**, ID420747.
- Iwai, T., Kaku, H., Honkura, R., Nakamura, S., Ochiai, H., Sasaki, T. and Ohashi, Y. (2002) Enhanced resistance to seed-transmitted bacterial diseases in transgenic rice plants overproducing an oat cell-wall-bound thionin. *Mol. Plant–Microbe Interact.* **15**, 515–521.
- Ji, H., Gheysen, G., Denil, S., Lindsey, K., Topping, J.F., Nahar, K., Haegeman, A., De Vos, W.H., Trooskens, G., Van Criekeing, V., De Meyder, T. and Kyndt, T. (2013) Transcriptional analysis through RNA sequencing of giant cells induced by *Meloidogyne graminicola* in rice roots. *J. Exp. Bot.* **64**, 3885–3898.
- Kim, S.T., Yu, S., Kang, Y.H., Kim, S.G., Kim, J.Y., Kim, S.H. and Kang, K.Y. (2008) The rice pathogen-related protein 10 (*JJOsPR10*) is induced by abiotic and biotic stresses and exhibits ribonuclease activity. *Plant Cell Rep.* **27**, 593–603.
- Kitanaga, Y., Jian, C., Hasegawa, M., Yazaki, J., Kishimoto, N., Kikuchi, S., Nakamura, H., Ichikawa, H., Asami, T., Ypshida, S., Yamaguchi, I. and Suzuki, Y. (2006) Sequential regulation of gibberellin, brassinosteroid, and jasmonic acid biosynthesis occurs in rice coleoptiles to control the transcript levels of anti-microbial thionin genes. *Biosci. Biotechnol. Biochem.* **70**, 2410–2419.
- Kogel, K.H., Ortel, B., Jarosch, B., Atzorn, R., Schiffer, R. and Wasternack, C. (1995) Resistance in barley against the powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei*) is not associated with enhanced levels of endogenous jasmonates. *Eur. J. Plant Pathol.* **101**, 319–332.
- Kreye, C., Bouman, B., Castañeda, A., Lampayan, R., Faronilo, J., Lactaoen, A. and Fernandez, L. (2009) Possible causes of yield failure in tropical aerobic rice. *Field Crops Res.* **111**, 197–206.
- Kyndt, T., Denil, S., Haegeman, A., Trooskens, G., De Meyer, T., Van Criekeing, W. and Gheysen, G. (2012a) Transcriptome analysis of rice mature root tissue and root tips in early development by massive parallel sequencing. *J. Exp. Bot.* **63**, 2141–2157.
- Kyndt, T., Nahar, K., Haegeman, A., De Vleeschauwer, D., Höfte, M. and Gheysen, G. (2012b) Comparing systemic defence-related gene expression changes upon migratory and sedentary nematode attack in rice. *Plant Biol.* **14**, 73–82.
- Kyndt, T., Vieira, P., Gheysen, G. and de Almeida-Engler, J. (2013) Nematode feeding sites: unique organs in plant roots. *Planta*, **238**, 807–818.
- Lessot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouzé, P. and Rombauts, S. (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* **30**, 325–327.
- Martin, F.J. and McInerney, J.O. (2009) Recurring cluster and operon assembly for phenylacetate degradation genes. *BMC Evol. Biol.* **9**, 36.
- Mitsuhara, I., Iwai, T., Seo, S., Yanagawa, Y., Kawahigasi, H., Hirose, S., Ohkawa, Y. and Ohashi, Y. (2008) Characteristic expression of twelve rice PR1 family genes in response to pathogen infection, wounding, and defense-related signal compounds (121/180). *Mol. Genet. Genomics*, **279**, 415–427.
- Mittler, R., Shulaev, V. and Lam, E. (1995) Coordinated activation of programmed cell death and defense mechanisms in transgenic tobacco plants expressing a bacterial proton pump. *Plant Cell*, **7**, 29–42.
- Muramoto, N., Tanaka, T., Shimamura, T., Mitsukawa, N., Hori, E., Koda, K., Otani, M., Hirai, M., Nakamura, K. and Imaeda, T. (2012) Transgenic sweet potato expressing thionin from barley gives resistance to black root disease caused by *Ceratocystis fimbriata* in leaves and storage roots. *Plant Cell Rep.* **31**, 987–997.
- Nahar, K., Kyndt, T., De Vleeschauwer, D., Höfte, M. and Gheysen, G. (2011) The jasmonate pathway is a key player in systemically induced defense against root-knot nematodes in rice. *Plant Physiol.* **157**, 305–316.
- Nahar, K., Kyndt, T., Nzogela, Y.B. and Gheysen, G. (2012) Absciscic acid interacts antagonistically with classical defense pathways in rice–migratory nematode interaction. *New Phytol.* **196**, 901–913.
- Nie, L., Peng, S., Bouman, B.A., Huang, J., Cui, K., Visperas, R.M. and Xiang, J. (2008) Alleviating soil sickness caused by aerobic monocropping: responses of aerobic rice to nutrient supply. *Field Crops Res.* **107**, 129–136.
- Nomura, K., Melotto, M. and He, S.Y. (2005) Suppression of host defense in compatible plant *Pseudomonas syringae* interactions. *Curr. Opin. Plant Biol.* **8**, 361–368.
- Onate-Sánchez, L., Anderson, J.P., Young, J. and Singh, K.B. (2007) AtERF14, a member of the ERF family of transcription factors, plays a nonredundant role in plant defense. *Plant Physiol.* **143**, 400–409.
- Ouyang, S., Zhu, W., Hamilton, J., Lin, H., Campbell, M., Childs, K., Thibaud-Nissen, F., Malek, R.L., Lee, Y., Zheng, L., Orvis, J., Haas, B., Wortman, J. and Buell, C.R. (2007) The TIGR rice genome annotation resource: improvements and new features. *Nucleic Acids Res.* **35**, D883–D887.
- Paine, J.A., Shipton, C.A., Chaggar, S., Howells, R.M., Kennedy, M.J., Vernon, G., Wright, S.Y., Hinchliffe, E., Adams, J.L., Silverstone, A.L. and Drake, R. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. *Nat. Biotechnol.* **23**, 482–487.
- Park, C.H., Kim, S., Park, J.Y., Ahn, I.P., Jwa, N.S., Im, K.H. and Lee, Y.H. (2004) Molecular characterization of a pathogenesis-related protein 8 gene encoding a class III chitinase in rice. *Mol. Cells*, **17**, 144–150.
- Peng, S., Bouman, B., Visperas, R.M., Castañeda, A., Nie, L. and Park, H.K. (2006) Comparison between aerobic and flooded rice in the tropics: agronomic performance in an eight-season experiment. *Field Crops Res.* **96**, 252–259.
- Pieterse, C.M., Leon-Reyes, A., Van der Ent, S. and Van Wees, S.C. (2009) Networking by small-molecule hormones in plant immunity. *Nat. Chem. Biol.* **5**, 308–316.
- Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S.C. (2012) Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* **28**, 489–521.

- Reversat, G., Boyer, J., Sannier, C. and Pando-Bahuon, A. (1999) Use of a mixture of sand and water-absorbent synthetic polymer as substrate for the xenic culturing of plant-parasitic nematodes in the laboratory. *Nematology*, **1**, 209–212.
- Sels, J., Mathys, J., De Coninck, B., Cammue, B. and De Bolle, M.F. (2008) Plant pathogenesis-related (PR) proteins: a focus on PR peptides. *Plant Physiol. Biochem.* **46**, 941–950.
- Shirasawa-Seo, N., Nakamura, S., Ukai, N., Honkura, R., Iwai, T. and Ohashi, Y. (2002) Ectopic expression of an oat thionin gene in carnation plants confers enhanced resistance to bacterial wilt disease. *Plant Biotechnol.* **19**, 311–317.
- Soriano, I.R., Prot, J.C. and Matias, D.M. (2000) Expression of tolerance for *Meloidogyne graminicola* in rice cultivars as affected by soil type and flooding. *J. Nematol.* **32**, 309–317.
- Stec, B. (2006) Plant thionins—the structural perspective. *Cell. Mol. Life Sci.* **63**, 1370–1385.
- Tandingan, I., Prot, J.C. and Davide, R. (1996) Influence of water management on tolerance of rice cultivars for *Meloidogyne graminicola*. *Fundam. Appl. Nematol.* **19**, 189–192.
- Thevissen, K., Ghazi, A., De Samblanx, G.W., Brownlee, C., Osborn, R.W. and Broekaert, W.F. (1996) Fungal membrane responses induced by plant defensins and thionins. *J. Biol. Chem.* **271**, 15 018–15 025.
- Van Buyten, E. and Höfte, M. (2013) *Pythium* species from rice roots differ in virulence, host colonization and nutritional profile. *BMC Plant Biol.* **13**, 203.
- Van Buyten, E., Banaay, C., Vera Cruz, C. and Höfte, M. (2013) Identity and variability of *Pythium* species associated with yield decline in aerobic rice cultivation in the Philippines. *Plant Pathol.* **62**, 139–153.
- Van Loon, L., Rep, M. and Pieterse, C. (2006) Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* **44**, 135–162.
- Ventura, W. and Watanabe, I. (1978) Growth inhibition due to continuous cropping of dryland rice and other crops. *Soil Sci. Plant Nutr.* **24**, 375–389.
- Vignutelli, A., Wasternack, C., Apel, K. and Bohlmann, H. (1998) Systemic and local induction of an Arabidopsis thionin gene by wounding and pathogens. *Plant J.* **14**, 285–295.
- Wan, X.R. and Li, L. (2006) Regulation of ABA level and water-stress tolerance of *Arabidopsis* by ectopic expression of a peanut 9-cis-epoxycarotenoid dioxygenase gene. *Biochem. Biophys. Res. Commun.* **347**, 1030–1038.
- Wasternack, C., Atzorn, R., Jarosch, B. and Kogel, K. (1994) Induction of a thionin, the jasmonate-induced 6 kDa protein of barley, by 2,6-dechloroisonicotinic acid. *J. Phytopathol.* **140**, 280–284.
- Yazaki, J., Kishimoto, N., Nagata, Y., Ishikawa, M., Fujii, F., Haahimoto, A., Shimbo, K., Shimatani, Z., Kojima, K., Suzuki, K., Yamamoto, M., Honda, S., Endo, A., Yoshida, Y., Sato, Y., Takeuchi, K., Toyoshima, K., Miyamoto, C., Wu, J., Sasaki, T., Sakata, K., Yamamoto, K., Iba, K., Oda, T., Otomo, Y., Murakami, K., Matsubara, K., Kawai, J., Carninci, P., Hayashizaki, Y. and Kikuchi, S. (2003) Genomics approach to abscisic acid- and gibberellin-responsive genes in rice. *DNA Res.* **10**, 249–261.
- Zhang, J., Xu, R., Elliott, M.C. and Chen, D.F. (1997) Agrobacterium-mediated transformation of elite indica and japonica rice cultivars. *Mol. Biotechnol.* **8**, 223–231.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Evaluation of gene expression by quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) and semi-quantitative RT-PCR. (a) *OsTHI7* + 6 expression in roots of

the T1 generation of *OsTHI7* + 6-overexpressing plants by qRT-PCR (top panel) and semi-quantitative RT-PCR (bottom panel). (b) Analysis of *OsPR1a* and *OsPR1b* expression in roots of the T1 generation of *OsTHI7* + 6-overexpressing plants by qRT-PCR. The gene expression level in qRT-PCR was normalized using two internal reference genes: *OsEXP* and *OsUBQ5*. Bars represent the mean expression level $\pm$ SE from two independent biological and three technical replicates, each containing a pool of eight plant roots. Data were analysed using REST2009 software. Significant differential expression ($P \leq 0.05$) is indicated by asterisks in comparison with empty vector roots (expression level = 1). The black horizontal line shows the expression level in empty vector plants (set at 1). RT-PCR was run for 25 cycles and *OsEXP* was used as a reference gene. WT, wild type; Vec, transgenic line containing an empty vector; OX, *OsTHI7*-overexpressing line.

Fig. S2 Shoot and root length of control and *OsTHI7*-overexpressing lines at 14 days after inoculation with *Meloidogyne graminicola* (28-day-old seedlings). Bars represent the mean $\pm$ SE of 24 plants. Different letters indicate statistically significant differences (Duncan's multiple range test with $P \leq 0.05$). Lowercase letters indicate significant differences in root length; uppercase letters indicate significant differences in shoot length. WT, wild type; Vec, transgenic line containing an empty vector; OX, *OsTHI7*-overexpressing line.

Fig. S3 Analysis of β -glucuronidase (GUS) activity in one *pOsTHI7::GUS* line after chemical treatment for 6 h and 3 days. Plant roots after 50 μ M abscisic acid (ABA) treatment (a, b), 100 μ M methyl jasmonate (MeJA) treatment (c, d) and 250 μ M benzo-1,2,3-thiadiazole carbothioic acid *S*-methyl ester (BTH) treatment (e, f). (g, h) Non-treated control roots. Scale bar, 0.5 mm.

Table S1 Differential expression patterns [$\log_2$ fold change (FC)] of rice thionin genes in *Meloidogyne graminicola*-infected giant cells (Ji *et al.*, 2013) and galls (Kyndt *et al.*, 2012a) at different time points after infection, as detected by mRNA-Seq, and in *Pythium graminicola*-infected roots at 2 dpi, as detected by microarray (GSE32582), in comparison with the uninfected control tissue. * $P \leq 0.1$; ** $P \leq 0.05$. Down-regulated genes, green; up-regulated genes, red; not differentially expressed, yellow. NE, not expressed in this tissue.

Table S2 Overview of the primers used in this study. Their GenBank accession/locus numbers (MSU7.0) are shown in parentheses.