Subcellular targeting of an evolutionarily conserved plant defensin MtDef4.2 determines the outcome of plant–pathogen interaction in transgenic Arabidopsis

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SUMMARY

The *Medicago truncatula* gene encoding an evolutionarily conserved antifungal defensin MtDef4.2 was cloned and characterized. *In silico* expression analysis indicated that MtDef4.2 is expressed in many tissues during the normal growth and development of *M. truncatula*. MtDef4.2 exhibits potent broad-spectrum antifungal activity against various *Fusarium* spp. Transgenic *Arabidopsis thaliana* lines in which MtDef4.2 was targeted to three different subcellular compartments were generated. These lines were tested for resistance to the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis* Noco2 and the hemibiotrophic fungal pathogen *Fusarium graminearum* PH-1. MtDef4.2 directed to the extracellular space, but not to the vacuole or retained in the endoplasmic reticulum, conferred robust resistance to *H. arabidopsidis*. Siliques of transgenic Arabidopsis lines expressing either extracellularly or intracellularly targeted MtDef4.2 displayed low levels of resistance to *F. graminearum*, but accumulated substantially reduced levels of the mycotoxin deoxynivalenol. The data presented here suggest that extracellularly targeted MtDef4.2 is sufficient to provide strong resistance to the biotrophic oomycete, consistent with the extracellular lifestyle of this pathogen. However, the co-expression of extracellular and intracellular MtDef4.2 is probably required to achieve strong resistance to the hemibiotrophic pathogen *F. graminearum* which grows extracellularly and intracellularly.

INTRODUCTION

Plant defensins are small cysteine-rich cationic proteins with four disulphide bonds. The three-dimensional structure of plant defensins is highly conserved, comprising three antiparallel β -strands and one α -helix. Despite their structural similarity, the

amino acid sequences of plant defensins are highly diverse (reviewed in Lay and Anderson, 2005; Thomma *et al.*, 2002). This variation in primary sequences may account for the myriad of different biological functions attributed to plant defensins (reviewed in Carvalho Ade and Gomes, 2009).

A large number of cationic plant defensins have been shown to inhibit the growth of various fungi at micromolar concentrations. This potent *in vitro* antifungal activity confers on these proteins the ability to provide resistance to fungal pathogens when expressed in transgenic plants (reviewed in Carvalho Ade and Gomes, 2009; Lay and Anderson, 2005; Murad *et al.*, 2007; Stotz *et al.*, 2009; Thomma *et al.*, 2002). Indeed, transgenic plants over-expressing defensins exhibit resistance not only to a range of hemibiotrophic and necrotrophic fungal pathogens (reviewed in Kaur *et al.*, 2011), but also to oomycetes (Portieles *et al.*, 2010; Zhu *et al.*, 2007).

The vast majority of plant defensins are synthesized as precursor proteins containing the secretory signal peptide sequence and the mature defensin peptide sequence. These defensins are predicted to be secreted to the extracellular space. However, a few floral defensins with an additional carboxy-terminal domain are targeted to the vacuole (Lay *et al.*, 2003). It is not known whether the subcellular localization of defensins plays an important role in plant defence against pathogens.

Previously, we have reported the presence of a multigene family encoding defensins in *Medicago truncatula* (Hanks *et al.*, 2005). One member of this gene family, designated MtDef4.2 (previously called MtDef4), encodes a protein that has a net positive charge of +6 (Sagaram *et al.*, 2011) and inhibits the growth of several filamentous fungi. For example, it inhibits the growth of *Fusarium graminearum* PH-1 with an IC₅₀ of 0.75–1.5 μ M (Ramamoorthy *et al.*, 2007). Here, we report the structure and expression analysis of the gene encoding MtDef4.2, which is predicted to be secreted to the extracellular space in *M. truncatula*. Transgenic Arabidopsis plants in which MtDef4.2 was directed to three different subcellular compartments were generated and characterized. We show that the subcellular localization of this defensin is a major determinant of its antifungal activity *in planta*.

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RESULTS

Medicago truncatula contains seven *MtDef4*-like genes

A multigene family encoding defensins has been reported previously in *M. truncatula* (Hanks *et al.*, 2005). One member of this gene family, represented by an expressed sequence tag (EST) TC85327 in the *M. truncatula* Gene Index (MtGI) version 7, encodes a defensin comprising a putative 29-amino-acid secretory signal peptide and a 47-amino-acid mature peptide (Fig. 1a), which was designated MtDef4.2 in the current study. The coding sequence of MtDef4.2 was isolated from *M. truncatula* genomic DNA through polymerase chain reaction (PCR) using the primers (Table S1, see Supporting Information) derived from the 5' and 3' ends of the nucleotide sequence of TC85327. Nucleotide sequence analysis of the 402-bp PCR product revealed the presence of a 170-bp intron interrupting the signal peptide sequence (Fig. 1b).

In MtGI version 11, TC85327 has been replaced by ESTs TC196370, TC195811, TC94214, TC1944213, TC195437 and TC187427. A further search of *M. truncatula* Gene Model, V3.5 version 3 and genome browser 2 identified six further homologues of MtDef4. Thus, the seven *MtDef4*-like genes identified are *Medtr3g032570.1* (*MtDef4.1*), *Medtr8g070770.1* (*MtDef4.2*), *Medtr8g070780.1* (*MtDef4.3*), *Medtr8g070790.1* (*MtDef4.4*), *Medtr8g075980.1* (*MtDef4.5*), *Medtr8g095370.1* (*MtDef4.6*) and

Medtr8g095375.1 (*MtDef4.7*). Amino acid sequence alignment of these defensins using CLUSTAL W2 is shown in Fig. 2. Defensin corresponding to TC85327 (*MtDef4*), identified in 2005 by our laboratory, is now denoted by gene ID *Medtr8g070770.1* or *MtDef4.2*. Of the seven defensin genes, *MtDef4.1* is present on chromosome 3 and the remaining six are located on chromosome 8 of *M. truncatula*. The predictive exon–intron arrangement of *MtDef4.1*–*4.7* genes is shown in Fig. 1b. Each gene encodes a mature peptide which is highly conserved, but not identical, in its amino acid sequence (Fig. 2).

Using the *M. truncatula* Gene Expression Atlas (MtGEA) version 2 (<http://mtgea.noble.org/v2/>), probeset IDs corresponding only to *MtDef4.1* (probeset ID Mtr.464.1.S1_at), *MtDef4.2* (probeset ID Mtr.7470.1.S1_at), *MtDef4.5* (probeset ID Mtr.18218.1.S1_at) and *MtDef4.6* (probeset ID Mtr.4093.1.S1_at) were identified. On the basis of these probeset IDs, *in silico* expression analysis of these genes was performed in various tissues and at different stages of seed maturity (Fig. 3). Thus, *MtDef4.2* was expressed at high levels with expression values ranging 4992–16673 in leaf, petiole, stem, vegetative bud, flower, pod and root, whereas the expression of *MtDef4.1*, *MtDef4.5* and *MtDef4.6* was very low in these tissues (Fig. 3a). During the seed-filling stage from 10 to 36 days after pollination (dap), expression of *MtDef4.2* was down-regulated, followed by a sharp increase in the seed coat (Fig. 3b). In contrast, *MtDef4.6* showed up-regulation of expression from 10 to 24 dap,

Fig. 1 Nucleotide sequence of *MtDef4.2* and the exon–intron maps of the *MtDef4*-like gene family in *Medicago truncatula*. (a) Nucleotide and deduced amino acid sequence of MtDef4.2. The signal peptide is highlighted in turquoise and the mature peptide is highlighted in red. (b) Exon–intron arrangement of the *MtDef4* gene family in *M. truncatula*. The size of each box is indicated in base pairs in parentheses.

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MtDef4.4  MARSIPLVSTIFVFFLLLVATEMGPIMVAE--ARTCETPSNNFKGLCVSDTNCASVCQTE 58
MtDef4.3  MARSVSLVSTIFVFFLLLVATEMGPSMVA---ARTCETPSNSFKGACFSDTNCASVCQTE 57
MtDef4.2  MARSVPLVSTIFVFFLLLVAT--GPSMVAE--ARTCESQSHKFKGFCASDHNCASVCQTE 56
MtDef4.1  MARSITLVCTIFFFLFLLVSTEMQPTHVEEPEARTCDSQSHSFKGVCIWKHNCAVCKTE 60
MtDef4.5  MALQFLSIRTIFLFLVLVATEMGSIMVVE--ARKCLSQSHSFKGLCLSDQNCATVCLTE 58
MtDef4.6  MER--KTLWFLFMLFLLAAD--IAVKTAE--GRRCESKSHKFKGPCSRDSNCASVCRGE 54
MtDef4.7  MER--KTLGILFMLFLVLAAD--VAVKTAE--GRRCESQSHKFKGFCVSDSNCGSVCRGE 54

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MtDef4.3  GFPGGHCCKGFRQRCFCITKPC--- 77
MtDef4.4  GFPGGHCEGFRQRCFCITKPC--- 78
MtDef4.2  RFSGGHCRGFRRRCFCITHC--- 76
MtDef4.1  GFTGGHCHGFRRCFCISKPC--- 80
MtDef4.5  GFTDGRGFRQRCFCISKPCLV 81
MtDef4.6  GFTGGDCRGFRRCFCITRNC--- 74
MtDef4.7  GFTGGDCRGVRHRCFCITRNC--- 74

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Fig. 2 Amino acid sequence alignment of the precursor MtDef4.1–4.7 defensins. Highly conserved cysteine residues involved in disulphide bridge formation are highlighted in yellow. Signal peptide is indicated in black italics.

Fig. 3 Expression analysis of defensins MtDef4.1, MtDef4.2, MtDef4.5 and MtDef4.6 based on MtGEA (*Medicago truncatula* Gene Expression Atlas). Expression in various tissues of *M. truncatula* (a) and at different stages of seed development and in the seed coat (b) is shown. dap, days after pollination.

followed by down-regulation in mature seed and seed coat (Fig. 3b). *MtDef4.1* and *MtDef4.5* did not show any appreciable levels of expression during seed filling and maturity.

Comparison of MtDef4.1–4.7 with defensins from other plants

Based on text mining (reviewed in de Beer and Vivier, 2008; Lay and Anderson, 2005), defensins sharing high sequence homology to MtDef4.1–4.7 were discovered in various legume and nonlegume plants. Alignment of the amino acid sequences of 28 MtDef4-like defensins was performed and a molecular phylogenetic tree was constructed using 1000 bootstrap trials (Fig. 4). Thus, MtDef4.2 clustered with *Helianthus annuus* defensin SD2, whereas MtDef4.1 and MtDef4.5 were closer to *Arabidopsis thaliana* defensin PDF2.5 (Thomma *et al.*, 2002). MtDef4.3 and

MtDef4.4 were closer to *Prunus persica* defensins AAL85480.1 and PdDfn1, and MtDef4.6 and MtDef4.7 clustered with *Ipomoea batatas* defensin with accession number AAS65426.1 (Fig. 4). Surprisingly, all seven MtDef4-like defensins have diverged from their counterparts in other legumes. Thus, none of the MtDef4.1–4.7 defensins clustered with Fabatin-1 and Fabatin-2 from *Vicia faba* or SE60 from *Glycine max* (Fig. 4).

MtDef4.2 inhibits the growth of several *Fusarium* spp. *in vitro*

When tested against *F. pseudograminearum* 9095, MtDef4.2 inhibited growth with an IC₅₀ value of 0.5–1.0 µM and complete inhibition at 3 µM (Table 1). The IC₅₀ values of MtDef4.2 were 1.2–1.5 µM and 1.3–1.8 µM against *F. proliferatum* and *F. verticillioides*, respectively (Table 1), with complete growth inhibition

Fig. 4 Neighbour-joining phylogram obtained using the amino acid sequences of MtDef4.1–4.7 and their homologues from other plants already published (reviewed in de Beer and Vivier, 2008; Lay and Anderson, 2005). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed using the DNASTar Lasergene program. The boxes indicate the position of *Medicago truncatula* defensins.

Table 1 *In vitro* antifungal activity of MtDef4.2 against three different *Fusarium* spp.

Fungus	Strain/isolate	IC ₅₀ (μM)*	LC (μM)†
<i>F. pseudograminearum</i>	9095‡ (NRRL 28062)	0.5–1.0	3.0
<i>F. proliferatum</i>	19§	1.2–1.5	4.0
<i>F. verticillioides</i>	ISU94445§	1.3–1.8	4.0

*IC₅₀, inhibitory concentration required for 50% mycelial growth inhibition.

†LC, lethal concentration required for complete inhibition of growth.

‡Isolate obtained from Fungal Genetics Stock Center, University of Missouri, Kansas City, MO, USA.

§Isolates obtained from Iowa State University, Ames, IA, USA.

observed at 4 μM for both species. These fungi showed a high degree of sensitivity to MtDef4.2, demonstrating the broad-spectrum antifungal activity of this defensin. MtDef4.2 did not induce any morphological distortion of fungal hyphae in any of the fungi tested, and thus appears to be a nonmorphogenic plant defensin.

Generation of transgenic Arabidopsis lines expressing extracellularly or intracellularly targeted MtDef4.2

In order to test the ability of MtDef4.2 to confer resistance to pathogens with contrasting lifestyles, we generated transgenic Arabidopsis lines in which the protein was directed either to the extracellular space or to the vacuole or retained in the endoplasmic reticulum (ER). For constitutive high-level expression of each gene, the figwort mosaic virus (FMV) 35S promoter was used. Three chimeric gene constructs, designated *MtDef4.2-Ec*, *MtDef4.2-Vc* and *MtDef4.2-ER*, were thus generated for the extracellular, vacuolar and ER localization of MtDef4.2, respectively, in transgenic plants (Fig. 5).

Twenty independent transgenic *A. thaliana* lines were generated for each of the three constructs. From these, five lines segregating 3:1 (indicative of a single insert) were selfed to obtain homozygous lines by progeny testing from the T₂ generation (Table S2, see Supporting Information). For each construct, three lines with correct transcript sizes (Fig. S1, see Supporting Information) were chosen arbitrarily for further characterization.

Expression analysis of MtDef4.2 in transgenic Arabidopsis lines

Quantitative reverse transcription (qRT)-PCR was used to determine the transcript level of the *MtDef4.2* gene in 5-week-old transgenic Arabidopsis seedlings. Compared with the wild-type (WT), transgenic *MtDef4.2-Ec* lines showed 100–291 000-fold higher expression of the gene (Fig. 6). *MtDef4.2-Vc* and *MtDef4.2-ER* lines showed 900–341 000-fold and 1.0–1800-fold higher expression of *MtDef4.2*, respectively (Fig. 6). Except for line ER12-2, all lines showed good expression of *MtDef4.2*.

Fig. 5 Expression vectors used for the subcellular targeting of MtDef4.2 in transgenic Arabidopsis. Chimeric *MtDef4.2* gene constructs were driven by the constitutive figwort mosaic virus (FMV) 35S promoter and terminated by nos-3'. The *MtDef4.2-Ec* construct directs the protein to the extracellular space. The *MtDef4.2-Vc* construct for vacuolar targeting and the *MtDef4.2-ER* construct for endoplasmic reticulum (ER) retention contain the barley lectin carboxy-terminal propeptide (CTPP) signal sequence and the KDEL sequence, respectively, fused in frame to the carboxy-terminal cysteine residue of mature defensin. nos, nopaline synthase.

Fig. 6 Expression analysis of *MtDef4.2* in 5-week-old seedlings of transgenic Arabidopsis lines using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Normalization was performed by comparing the expression of *A. thaliana* housekeeping gene *Ubi10* with respect to the wild-type (WT). Error bars represent the standard error (SE) of three replicates.

Next, we measured the MtDef4.2 protein in rosette leaves of 4-week-old plants and mature seeds of all transgenic lines using sandwich enzyme-linked immunosorbent assay (ELISA). In *MtDef4.2-Ec* lines, leaves and seeds expressed the protein at 0.7–1.8 and 0.1–5.1 μg/g fresh weight (FW), respectively (Table 2). *MtDef4.2-Vc* lines expressed the protein at 0.0–0.7 μg/g FW in leaves. However, the seeds of *MtDef4.2-Vc* lines contained a relatively larger quantity of 3.3–5.7 μg/g FW of protein (Table 2). Three *MtDef4.2-ER* lines expressed the protein at 0.1–0.9 and 0.0–3.1 μg/g FW in leaves and seeds, respectively (Table 2).

Subcellular localization of MtDef4.2 in transgenic Arabidopsis lines

Immunofluorescence was used to locate MtDef4.2 in paraffin sections of cotyledon cells of germinating seeds of transgenic

Table 2 Expression of MtDef4.2 in different tissues of wild-type (WT) and transgenic *Arabidopsis* lines as determined by sandwich enzyme-linked immunosorbent assay (ELISA).

Line	MtDef4.2 protein ($\mu\text{g/g}$ fresh weight $\pm$ SE*)	
	Leaves	Seeds
WT	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00
Ec4-3	1.8 $\pm$ 0.18	4.2 $\pm$ 0.21
Ec5-1	0.7 $\pm$ 0.29	0.1 $\pm$ 0.05
Ec15-5	1.5 $\pm$ 0.12	5.1 $\pm$ 0.18
Vc9-1	0.7 $\pm$ 0.33	5.7 $\pm$ 0.26
Vc25-8	0.01 $\pm$ 0.00	3.3 $\pm$ 1.10
Vc28-3	0.00 $\pm$ 0.00	4.5 $\pm$ 1.79
ER5-2	0.9 $\pm$ 0.27	1.3 $\pm$ 0.60
ER12-2	0.1 $\pm$ 0.05	0.0 $\pm$ 0.01
ER16-4	0.8 $\pm$ 0.36	3.1 $\pm$ 0.89

*Standard error calculated from three replicates.

Table 3 Spore counts (spore number/mL/2 plants $\times 10^4$) in wild-type (WT) and transgenic *Arabidopsis* leaves inoculated with *Hyaloperonospora arabidopsidis* Noco2 at 7 days post-inoculation (dpi).

Line	Mean $\pm$ SEM*	P
WT	13.42 $\pm$ 1.761	n.a.
Ec4-3	3.30 $\pm$ 0.505	<0.0001
Ec5-1	10.44 $\pm$ 3.695	0.4143
Ec15-5	5.94 $\pm$ 1.308	0.0012
Vc9-1	9.55 $\pm$ 1.138	0.0705
Vc25-8	14.32 $\pm$ 2.083	0.7435
Vc28-3	13.27 $\pm$ 2.630	0.9635
ER5-2	14.35 $\pm$ 2.672	0.7731
ER12-2	11.60 $\pm$ 2.585	0.5745
ER16-4	16.28 $\pm$ 1.661	0.2428

*Values show the mean of six biological replicates $\pm$ standard error of the mean (SEM). Analysed using Student's *t*-test.

lines. Extracellularly targeted MtDef4.2 in line Ec4-3 (Fig. 7a,b) showed accumulation in the cell wall, especially at the cell corners, with an intracellular signal indicating MtDef4.2 transport to the wall through the endomembrane system pathway. Line Vc9-1 showed a weak signal in vacuoles (Fig. 7c,d) and a punctate cytoplasmic signal that could represent endomembrane system components involved in transport to the vacuole or could be small vacuoles themselves. Line ER16-4 (Fig. 7e,f) showed MtDef4.2 in punctate structures interspersed with diffuse signal, consistent with the observation expected for a true ER location (Fig. 7g), considering the imperfect effects of the immunofluorescence preparation protocol. The live cell ER green fluorescent protein (GFP) signal in Fig. 7g shows that the large number of protein storage vacuoles and oil bodies constrict the ER to cytoplasmic regions between these structures, with the signal being strongest in cisternal junctions and around the nucleus. WT cotyledon cells showed no signal (Fig. S2, see Supporting Information).

MtDef4.2 directed to the extracellular space in transgenic *Arabidopsis* provides strong resistance to the obligate biotroph *Hyaloperonospora arabidopsidis* Noco2

Hyaloperonospora arabidopsidis Noco2 is a natural obligate oomycete pathogen of *A. thaliana* virulent on Col-0, which was used in this experiment. At 7 days post-inoculation (dpi), spore counts were calculated for WT and transgenic *Arabidopsis* plants. Spore counts were reduced significantly in transgenic plants expressing extracellularly targeted MtDef4.2 when compared with WT (Table 3), and this reduction was more pronounced in transgenic lines Ec4-3 and Ec15-5 than in line Ec5-1 (Table 3). The level of resistance observed in these lines was highly consistent with the expression level of MtDef4.2 in the leaf tissue of these lines (Table 2). Thus, line Ec4-3 showed the highest expression of MtDef4.2 (1.8 $\mu\text{g/g}$ FW) and hence the highest level of resistance to *H. arabidopsidis*. However, line Ec5-1 showed least resistance, consistent with the low expression of the protein (Table 2). No transgenic lines in which MtDef4.2 was directed either to the vacuole or retained in the ER showed any significant resistance to *H. arabidopsidis* as measured by the spore count compared with WT (Table 3).

Trypan blue staining of representative leaves from WT revealed conidiophores shedding abundant conidiospores, together with numerous hyphae (Fig. 8a). In line Ec4-3, a few hyphae were observed, but extensive trailing necrosis, indicative of a failed attempt at infection by the pathogen, was also seen (Fig. 8b), supporting the small number of spores observed in this line (Table 3). However, line Vc25-8 containing vacuolar MtDef4.2 and line ER16-4 containing ER-retained MtDef4.2 were no different from WT when examined for the presence of fungal hyphae and conidiophores (data not shown). Further, in order to examine the overall trend of spore counts in transgenic lines belonging to each targeting, data were subjected to analysis of variance (ANOVA) followed by Tukey analysis. As depicted in Figure 8c, MtDef4.2 directed to the extracellular space clearly conferred strong resistance to *H. arabidopsidis* when compared with the vacuolar or ER-retained protein (Fig. 8c).

Silques of transgenic *Arabidopsis* lines expressing extracellular, vacuolar or ER-retained MtDef4.2 exhibit low resistance to *F. graminearum*

As *F. graminearum* prefers to colonize the floral tissue at anthesis in small-grain cereals and produces the mycotoxin deoxynivalenol (DON) in association with host tissue, we tested the floral tissue of transgenic *Arabidopsis* lines for resistance to this pathogen. In our hands, we were able to achieve more reliable and consistent disease symptoms in silques than in flowers. We therefore chose to challenge silques of WT and transgenic plants with this pathogen

Fig. 7 Immunofluorescence labelling of MtDef4.2 in the seed of transgenic Arabidopsis lines. (a, b, inset) Line Ec4-3 targeting MtDef4.2 to the cell wall (arrowheads). Note the intracellular signal (arrows) showing protein being transported through the cell endomembrane system. (c, d, inset) Line Vc9-1 with vacuolar targeting. The signal is weak in the vacuoles, but can be seen in punctate structures (arrows) that could be small vacuoles or endomembrane system components transporting MtDef4.2 to the vacuoles. (e, f, inset) Line ER16-4 with MtDef4.2 retained in the endoplasmic reticulum (ER); arrows show the localization equivalent to that of ER-targeted green fluorescent protein (GFP) in cotyledon cells shown in (g). V, vacuoles; N, nucleus; scale bar for all panels, 5 µm.

to assess disease severity (DS) and DON accumulation. Point inoculation of apically wounded attached siliques with the macroconidia of *F. graminearum* PH-1 was employed to score DS using a scale of 0–7 as described by Cuzick *et al.* (2008), with slight modifications (Fig. 9). Because of space constraints, nine transgenic lines and WT were tested in three separate experiments in the same growth chamber, and the combined results are shown in Table 4. In each experiment, 150–250 siliques of transgenic lines and WT were challenged with macroconidia of *F. graminearum* and scored for DS at 3, 5 and 8 dpi. As reported by Urban *et al.* (2002), we also observed large variation in DS among the inoculated siliques of WT and transgenic lines. At 3 dpi, inoculated siliques of WT and transgenic lines were largely symptom free, except that a dark-brown necrotic ring at the point of inoculation was observed in some siliques. By 5 dpi, the necrotic ring had moved down through the silique, leaving behind light-brown bleaching. The siliques of lines Ec5-1, Vc9-1, ER5-2 and ER12-2 showed bleaching significantly lower down than that of WT (Table 4). However, DS on lines Ec4-3, Ec15-5, Vc25-8, Vc28-3 and ER16-4 was not as significant at 5 dpi (Table 4). At 8 dpi, the majority of infected siliques of WT plants

had DS of 3 and above whilst supporting the superficial growth of fungal hyphae, resulting in the drying of siliques. In contrast, siliques of all transgenic lines displayed significantly less DS, including transgenic lines Ec4-3, Ec15-5, Vc25-8, Vc28-3 and ER16-4, which showed improvement in DS over 5 dpi (Table 4). A relatively higher percentage of siliques with low DS (score of 0–2) were observed in transgenic lines than WT at 8 dpi (Fig. 10). Thus, siliques with a DS of 6 were observed in WT but not in transgenic lines Ec4-3, Vc28-3 and ER16-4 (Fig. 10). Normal senescence of siliques had set in after 8 dpi, making it difficult to differentiate fungus-induced bleaching from normal senescence; hence, plants were not scored beyond this time point. Siliques of WT and transgenic lines mock inoculated with deionized water were symptomless at all time points.

In an attempt to determine whether the observed visual resistance to *F. graminearum* correlated with reduced fungal growth, fungal biomass was observed visually in trypan blue-stained inoculated siliques of lines Ec4-3, Vc25-8 and ER16-4 and WT at 8 dpi. Representative inoculated siliques from these transgenic lines and WT were collected for trypan blue staining and the

Fig. 8 Transgenic *Arabidopsis* lines challenged with *Hyaloperonospora arabidopsidis* Noco2 at 7 days post-inoculation (dpi). (a) Representative leaves of wild-type (WT) and transgenic line Ec4-3. White arrows indicate fungal hyphae, black arrows conidiophores, red arrows trailing necrosis (indicative of failed infection attempt) and red asterisk conidiospores. Scale bar for both panels, 200 μm. (c) Analysis of variance (ANOVA) of spore count data followed by a Tukey analysis. Three transgenic *Arabidopsis* lines belonging to each construct were combined for this analysis. Different letters indicate statistically significant differences ($P < 0.05$).

remaining siliques were harvested to determine the fungal biomass using qPCR. Most of the fungal spores germinated and hyphae proliferated in the WT-inoculated siliques (Fig. 11a). Transgenic line Ec4-3 (Fig. 11b) was similar to WT in terms of microscopic symptoms. However, line Vc25-8 showed significantly more ungerminated fungal spores compared with WT (Fig. 11a,c). Much reduced hyphal growth, but abundant spores, were observed in line ER16-4 (Fig. 11d). These observations were further corroborated by fungal biomass data on total DNA isolated from the inoculated siliques. In WT siliques, 0.91 ng of fungal DNA/100 ng of total DNA (Fig. 11e) was measured. In contrast, transgenic lines Ec4-3, Vc25-8 and ER16-4 accumulated 0.88, 0.73 and 0.23 ng of fungal DNA/100 ng of total DNA, respectively (Fig. 11e). In particular, siliques of line ER16-4 accumulated approximately 75% less fungal DNA than WT.

Siliques of transgenic *Arabidopsis* lines expressing MtDef4.2 exhibit significantly reduced accumulation of DON on fungal challenge

DON levels in fungus-inoculated siliques from two independent experiments were measured at 8 dpi and the combined data are reported in Table 4. Although all transgenic lines showed reduced DON levels, line ER16-4 expressing ER-retained MtDef4.2 accumulated markedly reduced levels of DON (97% reduction) when compared with those in WT (Table 4). Another transgenic *Arabidopsis* line Ec5-1 accumulated 56% less DON compared with WT (Table 4).

Collectively, the data presented here reveal that the overexpression of either extracellularly or intracellularly targeted MtDef4.2 confers low levels of resistance to the hemibiotrophic fungus

Fig. 9 Modified scale of 0–7 used to score *Fusarium graminearum*-inoculated siliques: 0, normal; 1, bleaching of surface; 2, aerial mycelium growing on surface; 3, drying of most of the silique; 4, aerial mycelium growth accompanied by drying; 5, bleaching advancing to pedicle of silique; 6, mycelial growth on pedicle, occasional splitting of silique; 7, loss of silique by disease travelling down the stem and main stem constriction (not shown).

F. graminearum in the siliques of transgenic *Arabidopsis* lines. Although all transgenic lines accumulated reduced levels of DON, line ER16-4, expressing ER-retained MtDef4.2 (Table 2), accumulated substantially reduced levels of DON (Table 4). This striking reduction in DON level in the siliques of line ER16-4 also correlated with significantly reduced fungal biomass.

DISCUSSION

Here, we have characterized the *M. truncatula* gene encoding MtDef4.2 defensin (Hanks *et al.*, 2005) with broad-spectrum antifungal activity against various economically important *Fusarium* spp. (Table 1). Computational analysis revealed the presence of six additional MtDef4.2-like genes in *M. truncatula* (Fig. 2). MtDef4.1 is present on chromosome 3 and the remaining six genes MtDef4.2–4.7 are located on chromosome 8. MtDef4.2, MtDef4.3 and MtDef4.4 are present in close proximity to each other. Similarly, MtDef4.6 and MtDef4.7 are located very close to each other, indicating, perhaps, recent gene duplication. The MtDef4.2 homologues of *Arabidopsis*, PDF2.1, PDF2.3 and PDF2.6, also exist in a tandem array (Thomma *et al.*, 2002).

MtDef4.2 is expressed at high levels in different tissues, whereas MtDef4.1, MtDef4.5 and MtDef4.6 are expressed at very low levels (Fig. 3a). It remains to be determined whether the MtDef4.2 gene plays other important biological roles in *M. truncatula* in addition to plant defence. *Arabidopsis* defensins PDF2.1, PDF2.3 and PDF2.5 are also expressed in different tissues (Thomma *et al.*, 2002).

Defensins homologous to MtDef4.1–4.7 are present in many plants (Fig. 4) (reviewed in de Beer and Vivier, 2008; Lay and Anderson, 2005; Sagaram *et al.*, 2011). High homology of MtDef4.1–4.7 with defensins from other plants suggests that MtDef4.2 is evolutionarily conserved and that its homologues in *M. truncatula*, as well as other plants, probably play important biological roles.

Most plant defensins are targeted to the extracellular space (reviewed in Lay and Anderson, 2005), whereas a few defensins are vacuolar (Lay *et al.*, 2003). To our knowledge, no plant defensins are known to be retained in the ER. In this study, we tested the effectiveness of an antifungal plant defensin in conferring resistance to oomycete and fungal pathogens in transgenic plants when directed to different subcellular compartments. Our immunofluorescence results showed the expected localization for extracellularly targeted and ER-retained MtDef4.2 (Fig. 7a,b,e,f). We presume that MtDef4.2 peptide follows the typical behaviour of proteins synthesized in the ER. If it has a retention signal, it will remain in the ER lumen, as observed in line ER16-4 (Fig. 7e,f). MtDef4.2 without an ER retention signal will presumably be transported from the ER to its target compartments via the endomembrane system (Evert, 2006). In line Vc9-1, a weak MtDef4.2 signal was observed in the vacuole. Although the barley lectin vacuolar targeting signal used in our study has been shown previously to direct protein to the vacuole in tobacco (Bednarek *et al.*, 1990), it is likely that MtDef4.2 delivered to the vacuole might have been degraded in the vacuolar compartment, known for its lytic activity, and any that was not degraded would have been diluted in the relatively large volume of this compartment. Another possibility is that MtDef4.2 accumulated in a prevacuolar compartment, appearing as a punctate signal (Fig. 7c,d).

When tested against downy mildew pathogen *H. arabidopsidis*, transgenic *Arabidopsis* lines expressing extracellular MtDef4.2 exhibited strong resistance (Table 3), whereas transgenic lines in which protein was localized in the vacuole or retained in the ER did not (Table 3). Importantly, the level of resistance observed in transgenic lines with extracellularly targeted MtDef4.2 correlated well with the expression level of the protein (Table 2). These data are in accord with the obligate biotrophic lifestyle of this pathogen. Whilst growing inside the host, hyphae of *H. arabidopsidis* are located in the extracellular space (reviewed in Coates and Beynon, 2010) and the haustoria never break the cell plasma membrane. Thus, we postulate that MtDef4.2 present in the extracellular space is able to make direct contact with this pathogen and inhibit its growth. It will be of interest to determine whether this pathogen is severely restricted from forming haustoria and completing its life cycle in resistant lines.

The extracellularly and intracellularly targeted MtDef4.2 in transgenic lines provided low levels of resistance to *F. graminearum* (Table 4). The level of disease resistance achieved was not high, but the frequency of inoculated siliques with a DS of 0–2

Table 4 Disease severity and deoxynivalenol (DON) levels of wild-type (WT) and transgenic *Arabidopsis* siliques challenged with *Fusarium graminearum* PH-1.

Line	5 dpi			8 dpi			DON (ppm)†	
	Mean ± SEM*	<i>P</i>		Mean ± SEM*	<i>P</i>		Mean ± SEM*	<i>P</i>
WT	0.92 ± 0.040	—		2.99 ± 0.062	—		5.9 ± 2.10	—
Ec4-3	0.80 ± 0.027	0.5405		2.48 ± 0.063	3.5×10^{-16}		3.5 ± 2.60	0.2492‡
Vc28-3	0.65 ± 0.026	0.3230		2.37 ± 0.063	2.2×10^{-16}		4.2 ± 3.28	0.1508
ER16-4	0.84 ± 0.030	0.9698		2.52 ± 0.064	1.0×10^{-14}		0.2 ± 0.19	0.0119‡
WT	0.95 ± 0.025	—		3.05 ± 0.062	—		5.2 ± 1.18	—
Ec5-1	0.72 ± 0.020	3.5×10^{-9}		2.72 ± 0.055	1.2×10^{-6}		2.3 ± 0.52	0.0952
Vc9-1	0.69 ± 0.029	7.1×10^{-6}		2.71 ± 0.068	1.1×10^{-5}		3.0 ± 0.49	0.0749‡
ER12-2	0.79 ± 0.024	3.5×10^{-5}		2.65 ± 0.053	1.1×10^{-14}		3.5 ± 1.23	0.4206
WT	0.99 ± 0.039	—		3.09 ± 0.042	—		4.3 ± 1.92	—
Ec15-5	0.85 ± 0.031	0.3211		2.93 ± 0.038	3.0×10^{-11}		2.9 ± 1.74	1.000
Vc25-8	0.87 ± 0.031	0.3230		2.73 ± 0.042	2.2×10^{-16}		1.9 ± 1.69	0.1425‡
ER5-2	0.74 ± 0.032	5.45×10^{-5}		2.69 ± 0.051	2.2×10^{-16}		2.0 ± 1.22	0.4206

*Values show the mean of disease severity across two separate experiments ± standard error of the mean (SEM). Analysed using the Mann–Whitney test.

†DON levels were estimated in parts per million, determined at 8 days post-inoculation (dpi); data analysed using the Mann–Whitney test.

‡Approximate values, as program could not compute exact *P* values.

(resistant to moderately resistant) was significantly higher in transgenic lines than in WT (Fig. 10). Although all transgenic lines showed a reduction in DON levels compared with WT, the most significant reduction was observed in the transgenic line ER16-4. This dramatic reduction in the levels of DON in line ER16-4 was accompanied by significant reduction in the accumulation of fungal biomass and the presence of far fewer fungal hyphae (Fig. 11). Trypan blue-stained siliques of line ER16-4, however, showed abundant spores (Fig. 11d), perhaps because of an early switch from the vegetative to the sporulative growth phase. A thorough time course microscopy study will help to dissect this phenomenon in detail. These results demonstrate, for the first time, that ER-retained defensin is effective in conferring a low level of resistance to *F. graminearum*, but of reducing significantly mycotoxin accumulation in *Arabidopsis*.

Further molecular characterization of line ER16-4 is needed in order to unravel the precise mechanisms by which high expression of ER-retained MtDef4.2 provides resistance to DON accumulation. It will be interesting to determine whether the expression of genes encoding enzymes of the DON biosynthetic pathway (Desjardins, 2006) is dramatically reduced in the fungus by ER-retained MtDef4.2. The ER retention signal KDEL used in our study is expected to remain attached to MtDef4.2 and may influence its *in vitro* antifungal activity. Although the data presented here indicate that the fungal resistance observed in transgenic *Arabidopsis* lines is most probably a result of direct interaction of MtDef4.2 with the fungus, we cannot rule out the possibility that MtDef4.2 targeted to different subcellular compartments may cause changes in the expression of endogenous defence response genes in transgenic plants and determine the ultimate outcome of this plant–fungus interaction. Furthermore, differences in the pH values of different subcellular compartments might also influence

the charge and/or activity of MtDef4.2, thereby affecting the outcome of the plant–pathogen interaction. A full understanding of the mechanism of the antifungal action of MtDef4.2 is also needed to shed further light on the observed resistance to *F. graminearum* and the reduction in DON levels. Preliminary studies from our laboratory have shown that MtDef4.2 is internalized by the cells of *F. graminearum* and probably targets intracellular processes (U. S. Sagaram and D. M. Shah, unpublished data). As *F. graminearum* grows initially in the intercellular space and subsequently intracellularly during its life cycle in the host (Brown *et al.*, 2010; Bushnell *et al.*, 2003; Jansen *et al.*, 2005; Rittenour *et al.*, 2010), we are now in a position to test our initial hypothesis that the co-expression of extracellularly and intracellularly targeted MtDef4.2 in transgenic plants will provide more robust resistance to *F. graminearum* whilst completely eliminating mycotoxin accumulation. Thus, we have already begun to cross line ER16-4 with line Ec4-3 to test this hypothesis.

It should be noted that the three gene constructs used in this study for high-level extracellular or intracellular expression of MtDef4.2 caused no apparent morphological or developmental growth abnormalities in transgenic *Arabidopsis* lines. There were no significant differences in the leaf size and number, time to flowering and the ability to produce seeds when compared with WT plants. A more detailed phenotypic analysis of the transgenic lines will be required in order to uncover any subtle effects of transgene expression on the overall growth and development.

This is the first report, to our knowledge, in which the subcellular targeting of a plant defensin has been shown to play a significant role in determining the outcome of a plant–pathogen interaction. This study opens up new vistas for the genetic engineering of plants for resistance to fungal and oomycete pathogens using a plant defensin.

Fig. 10 Relative percentages of wild-type (WT) and transgenic siliques with different disease scores inoculated with *Fusarium graminearum* in three different experiments.

EXPERIMENTAL PROCEDURES

Biological material and growth conditions

Arabidopsis thaliana (Col-0) seeds were sown in potting soil (Fafard; superfine germinating mix, Earth City, MO, USA) and kept in a propagator for 2 days at 4 °C to ensure even germination. The pots of germinated seed were then moved to a controlled environment growth chamber with a temperature of 22 °C, 70% relative humidity and programmed for a 12-h light (150 µE/m²/s)/12-h dark cycle.

Hyaloperonospora arabidopsidis isolate Noco2, pathogenic on *A. thaliana* ecotype Col-0, was used in this study. Spores of Noco2 isolate were cultivated on 2-week-old Col-0 plants before collection. *Fusarium graminearum* strain PH-1 (NRRL31084) (Goswami and Kistler, 2005) was used for inoculation of transgenic *Arabidopsis* plants, and was kindly provided by Dr Jin-Rong Xu of Purdue University, West Lafayette, IN, USA. Strain PH-1 was routinely cultured in complete medium (CM) and, for conidiation, carboxymethyl cellulose medium was used (Ramamoorthy *et al.*, 2007).

Expression and purification of plant defensin MtDef4.2

Plant defensin MtDef4.2 was expressed in *Pichia pastoris* and purified from the growth medium as described previously (Ramamoorthy *et al.*, 2007). The molecular mass was confirmed using mass spectrometry.

In vitro antifungal activity of MtDef4.2

In vitro antifungal assays were performed as described previously (Spelbrink *et al.*, 2004). *Fusarium pseudograminearum* isolate 9095 (NRRL 28062), the causal organism of crown rot of wheat, was obtained from the Fungal Genetics Stock Center, University of Missouri, Kansas City, MO, USA. Dr Don White (University of Illinois, Urbana Champagne, IL, USA) kindly provided *F. proliferatum* isolate 19 and *F. verticillioides* isolate ISU94445. *Fusarium proliferatum* and *F. verticillioides* cause ear rot in maize and produce mycotoxins called fumonisins.

Cloning of MtDef4.2 and generation of plant expression vectors

The EST clone (TC85327), as reported previously (Hanks *et al.*, 2005), encodes a defensin designated as MtDef4.2. The full-length MtDef4.2 coding sequence was PCR amplified from the genomic DNA of *M. truncatula* 'Jemalong' A17 using the primers MtDef4.2-F (5'-ATGGTGGCAGAAGCAAGAA-3') and MtDef4.2-R (5'-CAAAGCATCTACGACGG-3'). The 402-bp PCR product was cloned into the pCR2.1-TOPO vector and the resulting plasmid was sequenced and designated as LP23. It encodes full-length MtDef4.2 consisting of a predicted 29-amino-acid secretory signal peptide and a 47-amino-acid mature peptide. It also contains an intron of 170 bp located within the signal peptide coding sequence. The native MtDef4.2 protein is predicted to be targeted to the extracellular space. LP23 plasmid was used as a template for the construction of chimeric genes for extracellular (Ec) targeting, vacuolar (Vc) targeting and endoplasmic reticulum (ER) retention of the MtDef4.2 protein. A 15-amino-acid vacuolar targeting sequence of the barley lectin (Bednarek *et al.*, 1990) and the Lys-Asp-Glu-Leu (KDEL) ER retention signal (Munro and Pelham, 1986, 1987) were added immediately after the last cysteine residue of MtDef4.2 using PCR. The primer sequences used for construction of the MtDef4.2-Ec, MtDef4.2-Vc and MtDef4.2-ER genes are shown in Table S1. The nucleotide sequences of all three chimeric genes were verified by sequencing, and the genes were subsequently cloned into the *Agrobacterium tumefaciens* binary expression vector AKK1472 (kindly provided by Dr Christopher Taylor, Ohio State University, Wooster, OH, USA) as

Fig. 11 Trypan blue-stained siliques inoculated with *Fusarium graminearum* and fungal biomass quantification at 8 days post-inoculation (dpi). (a) Representative siliques of wild-type (WT) and transgenic lines Ec4-3, Vc25-8 and ER16-4. White and yellow arrows point to fungal hyphae and spores, respectively. Scale bar for all panels, 50 μ m. (e) Fungal biomass was calculated in the inoculated siliques using quantitative polymerase chain reaction (qPCR).

NcoI-SacI fragments, such that the expression of each gene was driven by a strong constitutive 35S promoter of FMV (Maiti *et al.*, 1997; Sanger *et al.*, 1990), and terminated by a 3' flanking sequence of the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene.

Generation of transgenic Arabidopsis lines

All three constructs were introduced into the *Agrobacterium tumefaciens* strain GV3101 for plant transformation. *Arabidopsis thaliana* Col-0 plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected based on resistance to the herbicide Basta® (glufosinate). Basta®-resistant T₁ plants were screened for the presence of each gene by PCR, and expression of the MtDef4.2 protein was verified by ELISA. T₃ homozygous lines for each gene construct were obtained by progeny testing of the T₂ lines based on Basta® resistance, and the presence of each gene was confirmed by PCR. Three homozygous

lines each for the MtDef4.2-Ec, MtDef4.2-Vc and MtDef4.2-ER constructs were selected for further analysis (Table S2).

In silico expression analysis of defensin genes MtDef4.1–MtDef4.7

Medicago truncatula Gene Model, V3.5 version 3 and genome browser version 2 were used to identify homologues of MtDef4 and the corresponding gene IDs for these defensins. Further, MtGEA (Benedito *et al.*, 2008; He *et al.*, 2009) was queried for the expression of some of these genes in different organs of this plant. The MtGEA webserver hosts gene expression data compiled from 156 *Medicago* Affymetrix GeneChip hybridizations in 64 different experiments, covering a broad range of developmental and environmental conditions. As described in He *et al.* (2009), to make the 156 hybridizations cross-comparable, the raw chip data in .CEL format were either downloaded directly from the public

ArrayExpress repository (<http://www.ebi.ac.uk/arrayexpress/>) or exported from Affymetrix GeneChip Operating Software (GCOS) for experiments performed by the MtGEA project collaborators.

RNA isolation and cDNA synthesis

Total RNA was isolated from 5-week-old WT and transgenic Arabidopsis seedlings using the SV total RNA isolation system (Promega, Madison, WI, USA) following the manufacturer's instructions. Quantification of RNA was performed on a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and 2.0 µg equivalent of RNA was reverse transcribed into cDNA using the SuperScript® III first-strand synthesis system (Invitrogen, La Jolla, CA, USA).

qRT-PCR analysis

The cDNA samples from WT and transgenic Arabidopsis plants were diluted 1:10 in diethyl pyrocarbonate (DEPC)-treated water. Using Promega GoTaq® qPCR master mix, qRT-PCR assays were performed on the AB StepOne Plus-Real time PCR system (Applied Biosystems, Carlsbad, CA, USA) using gene-specific primers (Table S1), with the *A. thaliana ubiquitin10* (*At4g05320.2*) (Table S1) gene used as an internal control for gene expression analysis. The protocol used was as follows: initial denaturation and polymerase activation (95 °C for 5 min), amplification and quantification repeated 40 times (95 °C for 15 s, 60 °C for 10 s, 72 °C for 25 s with single fluorescence measurement), and melting curve programme (60–95 °C with fluorescence read every 0.3 °C). The data for transgenic Arabidopsis lines are presented as the relative quantity of gene expression normalized to the Arabidopsis housekeeping gene *Ubi10* and relative to WT.

ELISA

To determine MtDef4.2 expression in transgenic Arabidopsis lines, the sandwich ELISA method was developed and validated strictly as described in Allen *et al.* (2011). Polyclonal antibodies were raised against purified MtDef4.2 protein using services at the Animal Facility of Washington University, St. Louis, MO, USA. Total protein extracts were prepared by the homogenization of fresh rosette leaves (1:1 tissue to buffer ratio, w/v) and seeds (1:6 tissue to buffer ratio, w/v) in 50 mM tris(hydroxymethyl) aminomethane (Tris)/HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulphonyl fluoride, pH 7.6–8. Plant tissue extracts and a standard curve using purified MtDef4.2 protein were added in triplicate in each assay. The MtDef4.2 protein in micrograms per gram FW was calculated in the samples based on the standard curve, and the data were normalized to that of WT.

Subcellular localization of MtDef4.2 using immunofluorescence

Transgenic Arabidopsis seeds were surface sterilized and hydrated on a wet filter paper overnight at 4 °C. Hydrated seeds were then fixed in 4% formaldehyde in 50 mM piperazine-*N,N'*-bis(2-ethanesulphonic acid)

(PIPES) buffer (pH 6.8) for 2 h at room temperature and dehydrated in a graded ethanol series, followed by a graded Histoclear (National Diagnostics, Atlanta, GA, USA) series. They were infiltrated with a Histoclear/wax series (Spence, 2001) and embedded. Ten-micrometre sections mounted on slides were dewaxed in Histoclear and rehydrated for immunolabelling. The sections were blocked for 1 h at room temperature in 5% (w/v) nonfat dry milk plus 0.1% (v/v) Tween-20 in phosphate-buffered saline (PBS). They were then incubated at 4 °C overnight with MtDef4.2 primary antibody (10 µg/mL) in blocking buffer. After washing with PBS, slides were incubated with Alexa 488-conjugated goat anti-rabbit secondary antibody (diluted 1 : 100, Invitrogen) for 1 h at room temperature. After washing in PBS and deionized water, samples were mounted in anti-fading mounting medium [200 µg/mL *para*-phenylenediamine in 0.1 M Tris (pH 8.5) and 10% Mowiol (Calbiochem, La Jolla, CA, USA)]. Control tissue was incubated in secondary antibody only and no signal was detected (Fig. S2). Samples were imaged using a Zeiss LSM 510 confocal microscope (Zeiss, Jena, Germany) with a 40×, 1.2 NA water immersion, W Koor, C-Apochromat objective, employing an image pixel resolution of 130 nm or greater. Fluorescence was detected using 488 nm excitation (argon gas laser) and a BP 500–550 barrier filter. Maximum intensity projections were made using Imaris software (Bitplane AG, Zürich, Switzerland).

Hyaloperonospora arabidopsidis inoculations and spore count measurements

Spores of *H. arabidopsidis* Noco2 isolate were harvested from infected WT Arabidopsis plants and the concentration was adjusted to 5×10^5 spores/mL. Spore solution was sprayed onto 4-week-old transgenic and WT Arabidopsis plants, which were subsequently incubated in a growth chamber maintained at 17 °C for 7 days with a 10-h day : 14-h night cycle. The aerial parts of two plants from each line were cut and placed in a 50-mL tube containing 1 mL of sterile water. The tube was vortexed (about 15 s) and the spores were counted using a haemocytometer. For each transgenic and WT line, four technical replicates were evaluated in each replication and six biological repetitions were performed. Data were analysed using Student's *t*-test. Further, a combined ANOVA was performed for all three lines belonging to each construct and compared with WT using Tukey's test.

Inoculations of Arabidopsis siliques with *F. graminearum* and scoring of disease symptoms

For wound inoculation of siliques, the method described by Cuzick *et al.* (2008) was followed. Plants with several full-sized green siliques were selected and the stigmatic end of each silique was cut using sterilized scissors for inoculations. A 2-µL droplet of fungal spore suspension at a concentration of 10^6 spores/mL was placed on apically wounded siliques. Mock inoculations using deionized water spiked with 0.01% Tween-20 were performed in a similar manner. The infected siliques were scored on a scale of 0–7 (Fig. 9) as per Cuzick *et al.* (2008), with slight modifications, at 5 and 8 dpi. Photographs of infected siliques at different time points were taken using a dissecting microscope (Nikon SMZ1500, Melville, NY, USA) equipped with a QImaging Retiga 1300 camera (QImaging, Vancouver, BC, Canada). The Mann–Whitney test using R was employed for data analysis.

Trypan blue staining of inoculated leaves and siliques

Representative leaves of *Arabidopsis* transgenic lines and WT inoculated with *H. arabidopsidis* were cleared in fixative, stained using trypan blue and destained in chloral hydrate (Weigel and Glazebrook, 2002). Stained leaves were mounted with glycerol and microscopy was performed with a Nikon Eclipse E800 microscope equipped with a differential interference contrast apparatus (Nomarski optics), as described by Meinke (1994). Images were recorded with Openlab software (Improvision, Perkin Elmer, Waltham, MA, USA). A similar protocol was used for the staining and photographing of typical siliques of transgenic and WT *Arabidopsis* lines inoculated with *F. graminearum* collected at 8 dpi.

Quantification of *F. graminearum* DNA in silique tissue

In order to quantify *F. graminearum* biomass in inoculated siliques of transgenic and WT *Arabidopsis* lines, qPCR was performed. DNA was isolated from inoculated siliques of three transgenic lines Ec4-3, Vc25-8 and ER16-4, stored at -80°C , together with WT, using the cetyltrimethylammonium bromide (CTAB) protocol (Dietrich *et al.*, 2002). DNA (equivalent to 200 ng) was amplified using Promega GoTaq® qPCR master mix on an AB StepOne Plus-Real time PCR system using primers amplifying the *Tri5* gene (Boutigny *et al.*, 2009). *Tri5* encodes the enzyme trichodiene synthase which catalyses the cyclization of farnesyl pyrophosphate to trichodiene, the first step in trichothecene biosynthesis (Proctor *et al.*, 1995). The protocol used was similar to that employed for qRT-PCR. A five-point standard curve (in triplicate) starting with 50 ng of purified *F. graminearum* DNA spiked in WT *Arabidopsis* silique DNA, was used for the quantification. The amount of fungal DNA in transgenic and WT *Arabidopsis* lines was calculated from the cycle threshold (Ct) using the standard curve. The experiment was repeated twice following initial optimization.

DON analysis

Arabidopsis siliques challenged with fungus at 8 dpi were collected, weighed, quickly frozen in liquid nitrogen and stored at -80°C until processing. Quantitative analysis of DON was performed using the commercially available Ridascreen® Fast DON ELISA kit (R-Biopharm AG, Darmstadt, Germany) strictly following the manufacturer's instructions. The combined data from two independent experiments with three technical replicates each were analysed using the Mann–Whitney test.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Fig. S1 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) of wild-type (WT) and transgenic Arabidopsis seeds using gene-specific primers. The expected band sizes for MtDef4.2-Ec, MtDef4.2-ER and MtDef4.2-Vc lines are 311, 323 and 356 bp, respectively. 1, WT; 2, Ec4-3; 3, Ec15-5; 4, Ec5-1; 5, ER16-4; 6, ER5-2; 7, ER12-2; 8, Vc28-3; 9, Vc25-8; 10, Vc9-1. The Arabidopsis β -Tub4 gene (forward, CCTGATAACTTCGTCCTTGGG; reverse, GTTGTGGGATCCATTCCA; 1005 bp) was used as internal control.

Fig. S2 Immunofluorescence controls show no labelling. (a) Wild-type (WT) cotyledon cells labelled with primary and secondary antibodies; (b–d) cotyledon cells from Ec4-3, Vc9-1 and ER16-4 targeted MtDef4.2 plants labelled with secondary antibody only. Scale bar for all panels, 5 μ m.

Table S1 List of primers used for the characterization of transgenic Arabidopsis lines.

Table S2 Characterization of transgenic Arabidopsis lines overexpressing MtDef4.2 through various generations.

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