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Mutations in MyoB1 reduce pathogen susceptibility

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Use of enhancer trapping to identify pathogen-induced regulatory events spatially restricted to plant–microbe interaction sites

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SUMMARY

Plant genes differentially expressed during plant–pathogen interactions can be important for host immunity or can contribute to pathogen virulence. Large-scale transcript profiling studies, such as microarray- or mRNA-seq-based analyses, have revealed hundreds of genes that are differentially expressed during plant–pathogen interactions. However, transcriptional responses limited to a small number of cells at infection sites can be difficult to detect using these approaches, as they are under-represented in the whole-tissue datasets typically generated by such methods. This study examines the interactions between *Arabidopsis thaliana* (Arabidopsis) and the pathogenic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) by enhancer trapping to uncover novel plant genes involved in local infection responses. We screened a *β-glucuronidase* (*GUS*) reporter-based enhancer-trap population for expression patterns related to *Hpa* infection. Several independent lines exhibited *GUS* expression in leaf mesophyll cells surrounding *Hpa* structures, indicating a regulatory response to pathogen infection. One of these lines contained a single enhancer-trap insertion in an exon of At1g08800 (*MyoB1*, *Myosin Binding Protein 1*) and was subsequently found to exhibit reduced susceptibility to *Hpa*. Two additional Arabidopsis lines with T-DNA insertions in exons of *MyoB1* also exhibited approximately 30% fewer spores than wild-type plants. This study demonstrates that our enhancer-trapping strategy can result in the identification of functionally relevant pathogen-responsive genes. Our results further suggest that *MyoB1* either positively contributes to *Hpa* virulence or negatively affects host immunity against this pathogen.

Keywords: *Arabidopsis thaliana*, compatibility factor, disease susceptibility, *Hyaloperonospora arabidopsidis*, *MyoB1*, plant defence system, plant innate immunity.

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INTRODUCTION

A plant's active defence against pathogen attack often incorporates genetically regulated induced responses. The primary response is known as pattern-triggered immunity (PTI) (Chisholm *et al*., 2006; Jones and Dangl, 2006). In PTI, highly conserved regions of microbial molecules, termed microbial-associated molecular patterns (MAMPs), are recognized by pattern recognition receptors (PRRs) in the plant (Newman *et al*., 2013). Consequently, signal transduction pathways are activated, resulting in a defence response. In order to avoid plant immune responses, pathogens have evolved effector molecules that suppress plant defence reactions, attenuating PTI and resulting in host susceptibility (He *et al*., 2007; Ingle *et al*., 2006). In addition to a reduction in the plant immune response, such compatible interactions may involve microbial manipulation of other cellular mechanisms that promote pathogen colonization (Kamoun, 2007; Vogel *et al*., 2002). Viruses, for example, are known to recruit host translational machinery and chaperones to support aspects of the viral life cycle (Robaglia and Caranta, 2006; Verchot, 2012; Whitham and Wang, 2004; Yanga *et al*., 2014). Although the weakened PTI response during compatible interactions (termed basal defence) can often limit the growth of non-adapted pathogens, in many cases it does not prevent disease by adapted pathogens.

The plant's evolutionary response to pathogen effectors involves a secondary defence system, termed effector-triggered immunity (ETI) (Chisholm *et al*., 2006; Jones and Dangl, 2006), which is dependent on plant disease resistance (*R*) genes. *R* genes code for proteins that recognize, directly or indirectly, pathogen effectors. This *R*-mediated recognition instigates a faster, more robust defence response than PTI (Jones and Dangl, 2006).

Plant genes differentially expressed during plant–pathogen interactions can be important for host immunity or can contribute to pathogen virulence and fitness. Microarray analyses, RNA-seq and other large-scale transcript profiling methods have revealed the identity of many pathogen-responsive plant genes as well as the timing of their expression changes (Lodha and Basak, 2012; Zhu *et al*., 2013). However, they typically do not provide information regarding the spatial patterns of transcriptional reprogramming. Furthermore, these methods may often not allow for the

identification of genes that exhibit only weak or transient transcriptional changes or respond only in a small number of plant cells at infection sites. To overcome these limitations of standard transcript profiling approaches, we utilized enhancer trapping in *Arabidopsis thaliana* (Arabidopsis) to identify local events of differential gene expression triggered by a virulent isolate of the pathogenic oomycete *Hyaloperonospora arabidopsidis* (*Hpa*).

Enhancer trapping is a highly sensitive method for the detection of spatial gene expression patterns and has been used for the identification of the genes involved in plant development, stress responses and defence mechanisms (Atarés *et al*., 2011; Fridborg *et al*., 2004; Prasad *et al*., 2005). It is based on the random genomic integration of DNA constructs containing a core (or minimal) promoter fused to a reporter gene (Campisi *et al*., 1999; Springer, 2000). The core promoter is required, but insufficient, for reporter gene expression. Only when the enhancer trap integrates near an active enhancer can the reporter gene be expressed. Thus, detectable enhancer-trap action reflects the activity patterns of nearby endogenous enhancers and can reveal the expression patterns of genes controlled by these enhancers. Reporter gene activity patterns of enhancer-trap lines can display cells in which an enhancer is active.

In this study, we utilized Arabidopsis enhancer-trap lines to identify novel regulatory events at plant–microbe interaction sites. As a result, *MyoB1* (*Myosin Binding Protein 1*) was found to play a role in the susceptibility of Arabidopsis to *Hpa*. This gene may be involved in the host defence response or may be manipulated by *Hpa* effector proteins to enhance pathogen fitness or virulence.

RESULTS

Enhancer-trap screen reveals pathogen-related enhancer activity patterns

Hpa is an obligate biotrophic oomycete which forms a network of hyphae in Arabidopsis tissue during compatible interactions. Within seven days, the growth of *Hpa* results in the formation of sporangiophores (i.e. conidiophores), which are microscopic tree-like structures carrying asexual spores (Coates and Beynon, 2010). In order to retrieve nutrients from the host, *Hpa* utilizes specialized structures, called haustoria, which branch off from the hyphae, penetrate host cell walls and are surrounded by an extrahaustorial matrix and an extrahaustorial membrane (continuous with the plant plasma membrane) (Caillaud *et al*., 2014; Mims *et al*., 2004). Hyphae and haustoria are generally located in the leaf mesophyll, whereas sporangiophores emerge through stomata onto the surface of the leaf.

We screened more than 11,300 lines of an enhancer-trap population (Campisi *et al*., 1999) for local *Hpa*-induced β-glucuronidase (GUS) activity, focusing on gene regulatory events in a limited number of cells. These enhancer-trap lines contain a T-DNA construct with the *Cauliflower Mosaic Virus 35S* (*CaMV 35S*)-60 minimal promoter fused to the *uidA* (*GUS*) reporter gene in a Columbia-6 ecotype background (Campisi *et al*., 1999). The entire population was screened after inoculation with *Hpa* spores. The virulent isolate *Hpa*Noco2 was chosen as the infecting agent because of its robust growth on Columbia plants and its ability to induce basal defence responses in this host. *Hpa* does not have a gene that codes for GUS, and so the hyphae themselves are not stained during this assay. *Hpa*-induced GUS staining patterns in these enhancer-trap lines should reveal Arabidopsis cells in which the reporter gene is induced as a result of the activity of an endogenous pathogen-responsive enhancer. When *GUS* expression is limited to cells that are near or in contact with *Hpa* structures, the respective enhancer is probably restricted in its activity to pathogen infection sites. Consequently, genes controlled by such highly localized enhancer activity may be involved in a specific defence response or virulence-related process.

Three predominant infection-related histochemical staining patterns were identified in the screen: (i) a halo-like pattern surrounding hypersensitive response (HR)-like sites triggered by *Hpa*; (ii) enhanced *GUS* expression in guard cells surrounding emerging *Hpa* sporangiophores; and (iii) *Hpa* hyphae- and haustoria-associated staining in aerial tissue (Fig. 1). During HR, plant cells undergo programmed cell death, which is believed to stop the growth of pathogens that require live cells for their life cycle. This mechanism is typically observed during ETI; however, putative HR sites were also observed in our screen during compatible interactions in some enhancer-trap lines. The emergence of sporangiophores through stomata is an important morphological transition in the asexual life cycle of *Hpa*. However, guard cell staining was not exclusively associated with sporangiophores in all plants. As a result of the intimate association between hyphae/haustoria and plant cells, we chose lines with the corresponding staining pattern as our primary focus to uncover host genes at work in local Arabidopsis–*Hpa* interactions.

Screening was performed in two stages and various GUS staining patterns were seen throughout the process (Fig. S1, see Supporting Information). First, 113 separate pools, of 100 independent insertion lines each, were treated with *Hpa*Noco2. Approximately 40 whole seedlings from each pool were grown together in soil-filled pots and stained for *GUS* expression at 7 days post-infection (dpi). Pools containing individuals with promising staining patterns were purchased as ten-line lots for a second screen. To identify and isolate individual *Hpa*-associated response plants in the secondary screen, the ten-line lots were sown so as to contain twelve plants per 9 cm pot. Two-week-old seedlings were brushed with *Hpa*Noco2 spores to ensure maximum infection. At 7 dpi, two to three leaves and/or cotyledons containing sporangiophores were removed from each plant and stained for *GUS* expression. Individuals that exhibited *Hpa*-associated GUS staining in their

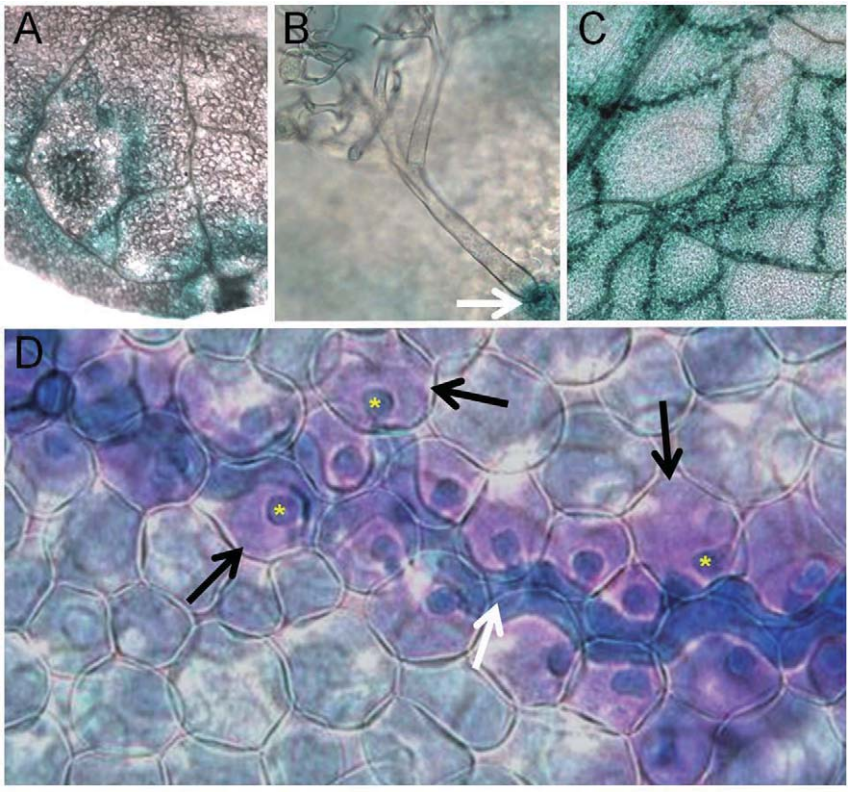

Fig. 1 Screening of an enhancer-trap population reveals three predominant pathogen infection-related histochemical staining patterns. (A) An X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) halo-like pattern (blue) surrounds putative infection sites. (B) An *Hpa*Noco2 sporangiophore emerges from a *myob1-2* X-gluc-stained (blue) stoma (white arrow). (C) Hyphae-associated X-gluc staining (blue) is seen in enhancer-trap plant leaves infected with the virulent isolate *Hpa*Noco2. (D) *Hyaloperonospora arabidopsidis* (*Hpa*)-infected *myob1-2* mesophyll tissue co-stained with trypan blue and MAGENTA-GlcA (van Damme *et al*., 2008), illustrating that responses of plant cells (magenta) are restricted to *Hpa* structures (dark blue). *Hpa* hyphae (white arrow) and haustoria (yellow asterisks) are stained with trypan blue and *β-glucuronidase* (*GUS*)-expressing plant cells are stained with MAGENTA-GlcA (black arrows).

leaves were assigned a letter of the alphabet for identification purposes and grown further to yield seeds. Progeny of these lines were used for experiments.

Seven independent lines of the screened population illustrated a strong and localized response to *Hpa* hyphae and haustoria in the secondary screen (Table S1, see Supporting Information). Progeny segregation ratios and insertion loci were verified in five of these lines (lines 'A', 'G', 'I', 'J' and 'K') (Table S1). Ligase-mediated polymerase chain reaction (LM-PCR) (Tsuchiya *et al*., 2009) was applied to identify the genomic regions flanking the respective enhancer-trap insertions in each line. Analyses of T-DNA selectable marker segregation were performed to examine each line with regard to the number of unlinked insertions and their genotypes at these loci. Lines 'A', 'I' and 'J', displaying the most robust staining surrounding *Hpa* hyphae and haustoria, were further characterized (Fig. S2, see Supporting Information).

One line, initially designated 'I' and subsequently named *myob1*-2, was found to be homozygous for a single insertion located within the coding region of *MyoB1* (At1g08800) (Fig. 2). GUS staining in *myob1*-2 is localized to leaf mesophyll cells associated with *Hpa* structures (Fig. 1D). Figure 1D shows *Hpa* hyphae and haustoria, stained with trypan blue, and the surrounding *myob1*-2 plant cells, stained for reporter gene expression with MAGENTA-GlcA. Additional *myob1*-2 staining patterns appeared to be associated with various root tissues (particularly during lateral root formation), one to three-week-old cotyledon, leaf and hypocotyl vasculature and floral organ abscission zones (Fig. S3, see Supporting Information). Such reporter gene staining patterns in *myob1*-2 indicate possible roles for this gene unrelated to pathogen interaction.

MyoB1 T-DNA mutants exhibit a reduction in *Hpa*Noco2 spore production

Given the specific hyphae/haustoria-associated staining pattern of *myob1*-2, we tested the enhancer-trap line in a susceptibility assay with *Hpa*Noco2. Two-week-old *myob1*-2 and plants of their parental wild-type background Col-6 were inoculated with *Hpa*Noco2. Pathogen growth was measured by the number of asexual spores produced on plants at 7 dpi. The *myob1*-2 plants displayed significantly fewer *Hpa*Noco2 spores than did Col-6 plants (Fig. 3). This reduced susceptibility was also seen in two additional independent Col-0 SALK lines with T-DNA insertions in *MyoB1* exons (named *myob1*-3 and *myob1*-4) (Figs 2 and 3).

The defence-associated enhancer-trap activity of *myob1*-2 reflects characteristics of an authentic *MyoB1*-associated enhancer

We have found previously that *MyoB1* transcripts exhibit mild but significantly enhanced levels in *Hpa*Noco2-infected whole Col-0 seedlings (see Discussion; Bhattarai *et al*., 2010). To confirm this

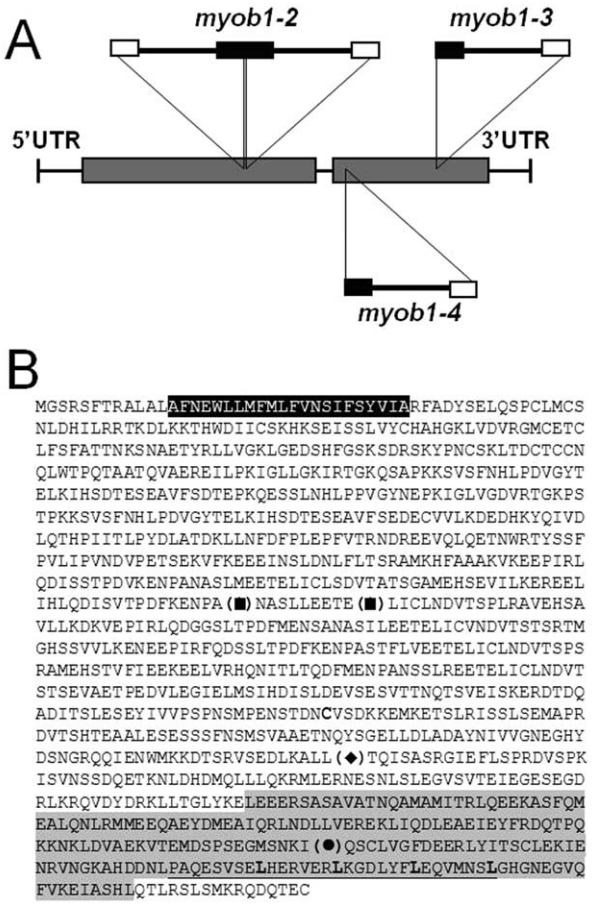

Fig. 2 Diagram representing *MyoB1* (*Myosin Binding Protein 1*) gene structure (A) and MyoB1 amino acid sequence (B). (A) Diagram illustrating the position and orientation of T-DNA insertions in *MyoB1*. Lines *myob1-3* (SALK_014285) and *myob1-4* (SALK_135666) contain an insertion in the second coding exon, whereas the insertion locus for *myob1-2* is in the first coding exon. Coding exons are shown as grey boxes. Black lines in the gene indicate 5' and 3' untranslated regions (UTRs) of the intron. T-DNA borders are represented by filled (right border) and open (left border) boxes. (B) The 1113-amino-acid sequence of MyoB1 based on The Arabidopsis Information Resource (TAIR) gene models At1g08800.1 and At1g08800.2. Putative coiled-coil structures (amino acids 906–988 and 1051–1080), predicted by EMBnet, PSORT and PROSITE, are underlined and the leucines of the leucine zipper are shown in bold. A putative transmembrane domain (amino acids 13–34), identified by the PHD program, is highlighted by a black background. A National Center for Biotechnology Information (NCBI)-predicted structural maintenance of chromosomes (SMC) region (amino acids 910–1097) is highlighted in grey. The *myob1*-2, *myob1*-3 and *myob1*-4 mutants are predicted to encode truncated proteins reaching up to '■', '●' or '◆', respectively. The region between the two '■' in *myob1*-2 was not present when T-DNA borders and their flanking genomic sequences were sequenced to determine the insertion locus. The T-DNA insertions are located in the area encoding for this deleted stretch.

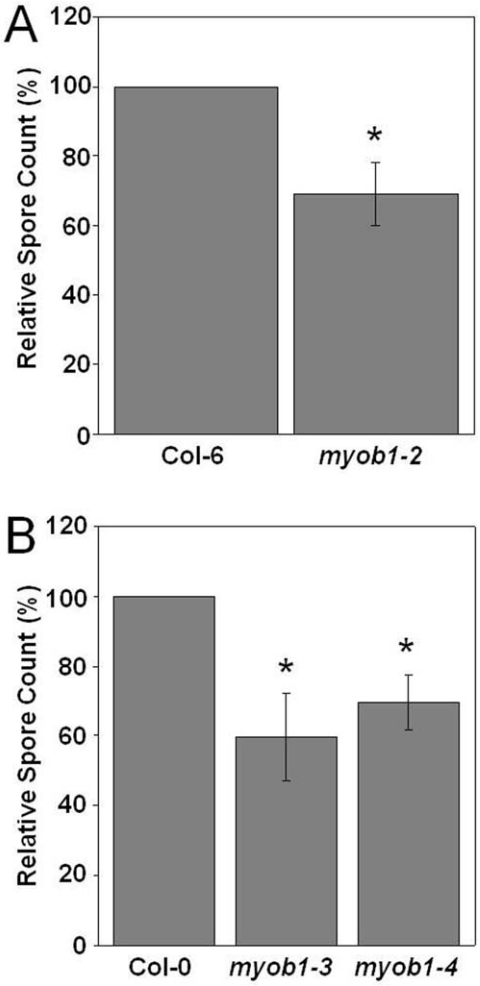

Fig. 3 Arabidopsis *myob1* mutants exhibit reduced susceptibility to a virulent isolate of *Hyaloperonospora arabidopsidis* (*Hpa*). Isolate *Hpa*Noco2 spore counts on *myob1* mutants were calculated as a percentage relative to wild-type controls for each individual experiment and then averaged. Student's *t*-test was used to determine statistically significant (*) differences between mutant and wild-type lines at a significance level of 5% or less. The error bars represent standard errors. (A) Enhancer-trap line, *myob1*-2, $P = 0.034$, $n = 11$. (B) Lines *myob1*-3 (SALK_014285), $P = 0.012$ and *myob1*-4 (SALK_135666), $P = 0.005$, $n = 5$.

expression trend, we tested wild-type Arabidopsis aerial tissue samples, heavily infected with *Hpa*, at 8 days post-treatment for the up-regulation of *MyoB1* transcripts using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Plants with a high density of sporangiophores on leaves, indicating a strong infection, were compared with water-only control-treated plants. Because *myob1-2 GUS* reporter gene expression was so closely associated with *Hpa* structures in leaves, only plants that were heavily infected were used. Cells showing *Hpa*-inducible *MyoB1* expression should be enriched in these samples. *Hpa*Noco2 infection induced a moderate, but reproducible, increase in *MyoB1* transcript levels in Col-0 leaves compared with

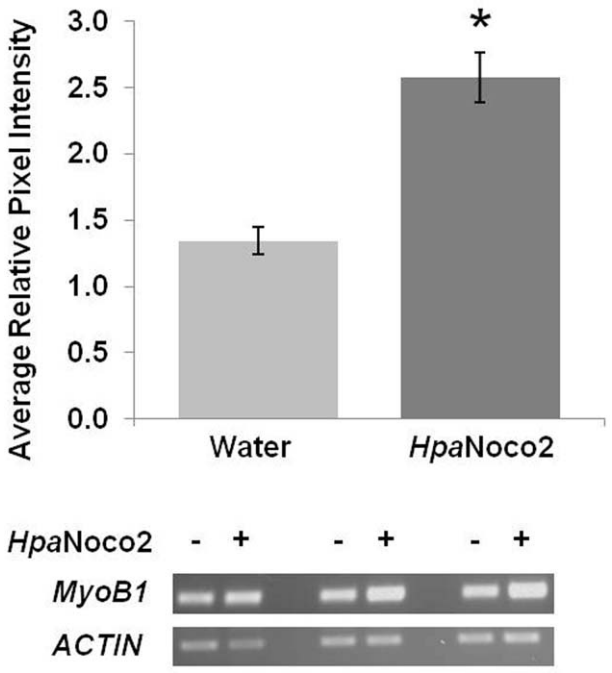

Fig. 4 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) shows an increase in *MyoB1* (*Myosin Binding Protein 1*) transcripts in Col-0 plants heavily infected with *Hpa*Noco2, at 8 days post-infection (dpi), compared with water-treated Col-0 plants. *MyoB1* RT-PCR band pixel intensity was quantified and divided by its corresponding *ACTIN* band intensity. The relative pixel intensity for three biological replicates for each treatment group was averaged using Image J software. Student's *t*-test was used to calculate significance (*), $P = 0.010$. The gel photograph represents three independent biological replicates that were used for the graph above.

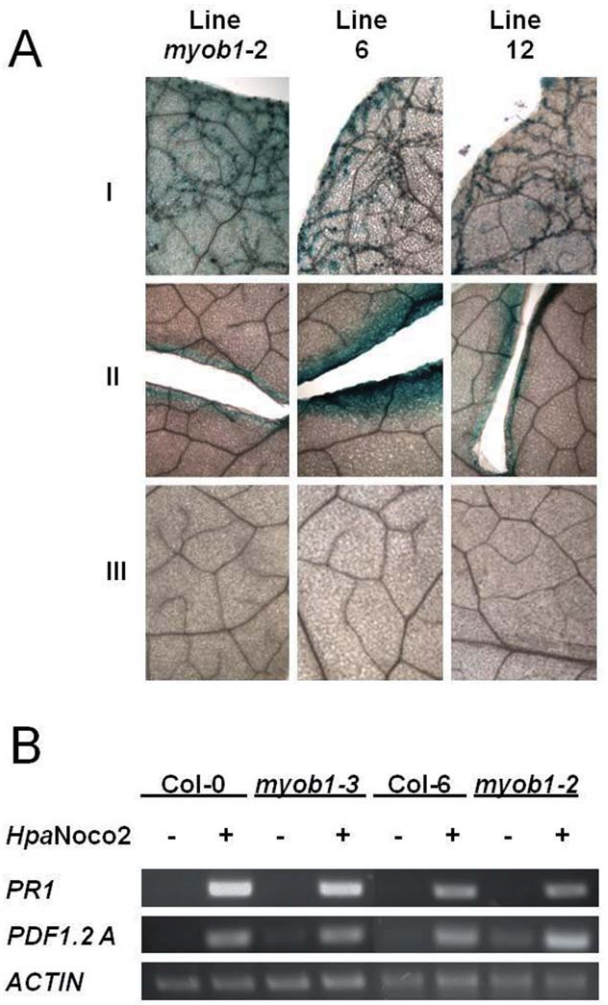

Fig. 5 A *MyoB1* (*Myosin Binding Protein 1*) promoter-controlled reporter gene shows a response to oomycete infection and wounding, yet canonical plant defence pathway marker genes are not influenced directly by *MyoB1*. (A) *MyoB1* promoter:*GUS* fusion transgenic lines mimic the staining patterns seen in the original enhancer-trap line (*myob1-2*). Two independent *pMyoB1*:*GUS* lines (lines 6 and 12) and *myob1*-2 are shown at 7 days post-infection (dpi) with the virulent isolate *Hpa*Noco2 (I), at 48 h post-wounding (II) and untreated (III). Post-treatment, plants were placed in a solution containing X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid), followed by the removal of chlorophyll with ethanol. (B) *PR1* and *PDF1.2A* are not constitutively transcribed in *myob1* mutant plants. A single representative semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) experiment shows transcripts from plants heavily infected with *Hpa*Noco2 at 7 dpi (+) and from water-treated (–) plants.

water-treated controls (Fig. 4). Thus, the *Hpa*Noco2 enhancer-trap activity observed in *myob1-2* plants appears to represent an authentic transcriptional response of the endogenous *MyoB1* gene.

To verify that the reporter gene expression pattern seen in *myob1-2* plants is mediated by an enhancer in the *MyoB1* promoter region, we generated transgenic *pMyoB1*:*GUS* lines that contained a construct with the entire 2143-bp *MyoB1* upstream intergenic promoter region fused to the *GUS* reporter gene. These lines were tested for the responsiveness of their reporter gene to *Hpa*Noco2 infection. Of ten tested independent T1 *pMyoB1*:*GUS* lines, nine exhibited an *Hpa*Noco2-associated staining pattern similar to *myob1-2*. Thus, the *MyoB1* promoter region harbours the enhancer responsible for *GUS* activity in *myob1*-2 plants. In addition, *pMyoB1*:*GUS* transgenic lines mimic responses by the original enhancer-trap line when exposed to wounding (Fig. 5A). Two *pMyoB1*:*GUS* lines and *myob1-2* that were wounded or infected with *Hpa*Noco2 are shown in Fig. 5. Surprisingly, neither *myob1*-2 nor *pMyoB1*:*GUS* lines exhibited a clear *GUS* expression response to the exogenous application of salicylic acid (SA), the SA analogues 3,5-dichloroanthranilic acid (DCA) and 2,6-dichloroisonicotinic acid (INA) or the biologically active jasmonic acid (JA) derivative methyl jasmonate (data not shown).

DISCUSSION

Previously, we have used microarrays to study transcriptome changes triggered by *Hpa* in whole Arabidopsis seedlings

(Bhattarai *et al*., 2010; Eulgem *et al*., 2004, 2007). These studies enabled us to gain a comprehensive overview of the types of genes differentially expressed in response to infections with avirulent and virulent isolates of this obligate biotroph. Comparisons between responses in susceptible host backgrounds with those in resistant ones further allowed us to identify the genes potentially contributing to plant immunity. Reverse genetic studies performed using Arabidopsis T-DNA mutants confirmed some of our predictions (Knoth *et al*., 2007; Knoth and Eulgem, 2008). Several other laboratories have performed similar studies on responses triggered by *Hpa* or other pathogen species in whole-plant tissues or aerial plant parts (Chen *et al*., 2002; Maleck *et al*., 2000; Sato *et al*., 2007; Tao *et al*., 2003). Although such approaches proved to be powerful with regard to the global overview of differential expression events detected, they may be insufficient to identify transcriptional changes specific to highly localized defence responses. Microarray studies and similar genome-wide transcript profiling approaches, which are typically performed with complex tissue samples, may not be sufficiently sensitive to detect transcriptional events occurring in only a few cells. Enhancer trapping, however, can pinpoint responses in small numbers of plant cells.

In our study, enhancer trapping revealed a plant gene affecting the outcome of interactions with virulent *Hpa* that had previously been overlooked in microarray analyses. According to ATH1 GeneChip data, *Hpa*Noco2 triggers a significant 1.3-fold up-regulation of *MyoB1* transcript levels in whole Col-0 seedlings (Bhattarai *et al*., 2010). However, because of its weak extent, this differential expression response was not considered to be important and was not followed up by further functional studies.

Transient gene expression changes can also be easily missed in microarray and mRNA-seq studies where, because of the high cost of these experiments, only a limited number of time points can be examined. However, transient gene expression changes can be robustly detected by *GUS*-containing enhancer traps because of the long half-life of the encoded GUS enzyme, which is stable for days (de Ruijter *et al*., 2003). In addition, time points as late as that chosen for our screen (7 dpi) had not been included in our previous microarray studies on *Hpa* responses (Bhattarai *et al*., 2010; Eulgem *et al*., 2004, 2007). Thus, the conditions used in our enhancer-trap screen specifically allowed for the identification of gene regulatory events that are highly localized, transient, weak in extent and/or late, theoretically bypassing four deficiencies of our previous microarray studies.

Arabidopsis–*Hpa* interactions have been studied since the early 1990s (Holub *et al*., 1993; Slusarenko and Schlaich, 2003; Tör *et al*., 1994). Although it is not well understood how host metabolites are transferred into the haustorial cytoplasm of this obligate biotrophic parasite, several studies have indicated that *Hpa* effectors are transferred into the host cells via haustoria (Birch *et al*., 2006; Whisson *et al*., 2007). Obligate biotrophs are unable to meet their nutritional needs without their host(s) (Baxter *et al*., 2010). Studies have shown that Arabidopsis mutants deficient in specific amino acid homeostasis are more resistant to virulent *Hpa* (van Damme *et al*., 2009; Stuttmann *et al*., 2011), implying a reliance on the plant to provide certain amino acids for the pathogen. *GUS* expression in *myob1*-2 and *pMyoB1*:*GUS* plants is tightly associated with hyphae and haustoria (Figs 1D and 5A), implicating *MyoB1* activity in response to these *Hpa* structures. In addition, *MyoB1* transcripts are up-regulated in heavily *Hpa*Noco2-infected tissue. If haustoria are key interaction sites with plant cells, it is possible that *MyoB1* is manipulated by *Hpa* at these locations in a manner that supports pathogen growth. A role of MyoB1 in supporting the transfer of critical metabolites from the host to *Hpa* at haustoria would be consistent with the phenotype of *myob1* mutants, which exhibit reduced *Hpa* susceptibility. In this case, MyoB1 would serve an *Hpa* fitness-associated function and act as a host compatibility factor.

Alternatively, *MyoB1* may be a component of the plant immune system. The fact that *Hpa* susceptibility is reduced in *myob1* mutants may point to a role of this gene as a negative regulator of defence. Plant immune responses are controlled by a complex network of regulatory processes (Katagiri, 2004; Sato *et al*., 2010; Tsuda *et al*., 2009). Two major regulators of defence gene expression, the phytohormones SA and JA, work alone or in combination to activate defence genes through this network (Derksen *et al*., 2013). Compatible host interactions with biotrophic pathogens and mechanical wounding are typically associated with the SA and JA defence pathways, respectively. Both *Hpa*Noco2 infection and wounding induce reporter gene expression in *myob1*-2 and *MyoB1* promoter:*GUS* fusion transgenic lines (Fig. 5A). However, disruption of *MyoB1* coding exons does not seem to result in enhanced transcript levels of the established SA or JA pathway maker genes, *PR1* and *PDF1.2A*, respectively (Figs 5B and S4, see Supporting Information), indicating that *MyoB1* is not likely to be a negative regulator of SA- or JA-mediated defence responses. In addition, three independent experiments comparing *Pseudomonas syringae* growth in aerial tissues of *myob1* mutants and Col-0 indicated that *MyoB1* does not appear to have an effect on resistance against this bacterial pathogen (Fig. S5, see Supporting Information). Thus, *MyoB1* is unlikely to serve as a general regulator of immune responses. Taken together, our results strongly suggest that *MyoB1* is a compatibility factor rather than a component of the plant immune system.

Previous studies have found host factors that affect susceptibility to pathogens without involving known defence responses. The Arabidopsis lignin biosynthesis mutant *comt1* shows reduced asexual *Hpa* sporulation and increased oospore formation without activating *PR1* and *PDF1.2*, suggesting that changes in plant

physiology and lignin composition can affect pathogen growth (Quentin *et al*., 2009). A comparison of the resistance of Arabidopsis accessions to powdery mildew has identified various host compatibility factors involved in the maintenance of pathogen growth and compatibility, especially in obligate biotrophs (Fabro and Alvarez, 2012). *Myob1*-2 GUS staining patterns unrelated to pathogen interaction (Fig. S3) also suggest that *MyoB1* is a compatibility factor, in that it has roles associated with plant maintenance that are putatively manipulated by *Hpa* to assist in its own perpetuation.

MyoB1 is a novel component of genetic mechanisms operating during the compatible interaction between *Hpa* and Arabidopsis. Its specific role is unknown. Previous publications have pointed to various putative roles for the protein, including stress responses (Gong *et al*., 2001; Stecker *et al*., 2014) and development (Ikeda *et al*., 2006). Recent work has uncovered a contribution to development by MyoB1 together with MYOSIN XI proteins (Peremyslov *et al*., 2013). The same study determined that MyoB1 is a myosin receptor and a major component of a distinct vesicular transport mechanism (Peremyslov *et al*., 2013). Similar to *myob1* mutants, *agd5* (involved in membrane trafficking) knockout lines also show reduced susceptibility to *Hpa*Noco2 (Schmidt *et al*., 2014). *AGD5* regulates trafficking processes related to cell separation (Liljegren *et al*., 2009) and has comparable attributes to *MyoB1*, in that their proteins are both predicted to be myristoylated at the N-terminus, a modification that promotes membrane interaction (Biosson *et al*., 2003).

The vesicular transport mechanism described by Peremyslov *et al*. (2013) involves the binding of MyoB1 to class XI myosins. Myosin mutants tested against adapted and non-adapted fungal pathogens exhibited compromised cell wall penetration resistance and enhanced disease susceptibility (Yang *et al*., 2014). The study pointed to roles for myosins as integral components of plant cell defence responses against fungi (Yang *et al*., 2014). Although disruption of class XI myosins leads to increased disease susceptibility to fungi, whereas *myob1* mutants show reduced susceptibility to an oomycete pathogen, both of these components of a specific vesicular trafficking pathway respond to pathogen infection. Future studies may further illustrate the diversity of specific vesicular trafficking mechanisms in response to a wide variety of plant pathogens.

Additional studies could focus on other MyoB family members. We tested three *MyoB1* paralogs (using T-DNA insertion SALK lines interrupting coding exons) in susceptibility assays with the virulent isolate *Hpa*Noco2. Like the *myob1* mutants tested, SALK_074471C (in At1g74830) showed significantly reduced susceptibility to the pathogen relative to wild-type plants (Fig. S6, see Supporting Information); however, SALK_042878C (in At5g16720) and SALK_086199C (in At5g06560) did not (Fig. S6).

Our study focused on local plant defence responses, not only because they may be under-represented in large-scale genomic studies, but also because they could provide key information about spatially controlled, highly specific gene regulatory mechanisms involved in plant–microbe interactions. Although, at this point, the role of *MyoB1* in affecting the outcome of Arabidopsis–*Hpa* interactions is unclear, our findings point to a possible link between *Hpa* virulence activities and host vesicle transport in cells closely associated with *Hpa* haustoria.

EXPERIMENTAL PROCEDURES

Plant material, pathogen infections and tissue staining

Uninfected Arabidopsis plants were grown on soil under fluorescent light (16-h day and 8-h night, 21 °C, 100 $\mu Em^{-2}s^{-1}$). After *Hpa* infection, plants were grown under conditions conducive to *Hpa* growth (10-h day and 14-h night, 17 °C, 100 $\mu Em^{-2}s^{-1}$). Plants used in this study were in either the Col-0 or Col-6 ecotype background. Two-week-old seedlings were used for all *Hpa* infections and other defence-related treatments. *Hpa* was propagated and applied to Arabidopsis as described previously (McDowell *et al*., 2000). T4-generation Thomas Jack Arabidopsis enhancer-trap lines (http://www.dartmouth.edu/~tjack/et.html) containing T-DNA insertions derived from the pD991 vector were obtained from the Arabidopsis Biological Resource Center (ABRC, http://abrc.osu.edu/). The initial screen included the entire population (113 100-line pools) spray inoculated with *Hpa*Noco2 (3×10^4 spores/mL of water). Lots CS19772-CS19781, CS19802-CS19811 and CS30741-CS30750 were used in the secondary screen. A Leica DM/LB2 (Leica, Wetzlar, Germany) microscope equipped with an RT colour SPOT camera was used to visualize and image the leaves.

SALK_014285 and SALK_135666 (homozygous for insertions in exons of At1g08800) were obtained from the ABRC and used in *Hpa* infection assays. Two-week-old seedlings were spray inoculated with *Hpa* spore suspensions (*Hpa*Noco2, 3×10^4 spores/mL of water, 2 mL per 9 cm square pot) with (Preval, Coal City, IL USA) sprayers. *Hpa* growth levels were determined at 7 dpi using a hemocytometer to calculate the spore density of a suspension of 20 mg of fresh weight of infected aerial tissue in 10 mL of water. Student's *t*-test was used to determine whether the effects of the *myob1* mutants on sporulation were statistically significant.

Histochemical GUS staining was performed by soaking tissues for 18 h in a 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid-containing [X-gluc (1 mg/mL; Gold Biotechnology, Inc., St. Louis, MO, USA) or MAGENTA-GlcA (0.5 mg/mL; Duchefa Biochemie, Haarlem, the Netherlands)] solution (50 mM Na_2PO_4, pH 7.2, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, 0.05% Triton-X) at 37 °C and cleared with 95% ethanol. Trypan blue dye was used to stain *Hpa* structures, as described previously (McDowell *et al*., 2000).

Pseudomonas syringae DC3000 infections were performed as described previously (Tornero and Dangl, 2001).

LM-PCR and RNA transcript analysis

Total DNA was isolated from aerial plant tissue using alkyl-trimethyl-ammonium bromide (CTAB) extraction buffers I [0.2 M Tris-HCl, pH 7.5,

1.4 M NaCl, 0.02 M ethylenediaminetetraacetic acid (EDTA)] and II (0.05 M CTAB), and was performed as described previously (Doyle and Doyle, 1987). LM-PCR was performed as described previously (Tsuchiya *et al*., 2009). The primers used to amplify nested left border (LB) and right border (RB) sequences (within the pD991 vector) were LB-LMPCR-1 (5'-CGGT CTCAATGCAAAAGGGGAACG-3'), LB-LMPCR-2 (5'-ATAATAACGCTGCGG ACATCTACATTTTGG-3'), RB-LMPCR-1 (5'-GTCGTGATGGGAAAACCTGG CGTTAC-3') and RB-LMPCR-2 (5'-GCACCTGAATGGCGAATGAGACCTC-3').

For segregation analysis to verify transgene homozygosity, T5 seeds were surface sterilized with 75% ethanol–0.05% Triton-X, rinsed in 100% ethanol, washed in sterile water and sown on plates (2.1 g/L Murashige and Skoog salts and agar) containing 50 μg/mL kanamycin. PCR genotyping was performed as described previously (Alonso *et al*., 2003).

Total RNA was isolated from infected and uninfected seedlings using TRIZOL (Ambion, Carlsbad,CA, USA). Total RNA (1 μg) was incubated with 1 unit of DNase I (Fermentas, Glen Burnie, MD, USA). After inactivation of DNase I, RNA was reverse transcribed using 200 units of Maxima reverse transcriptase (Fermentas). PCR cycle numbers were 28 for *MyoB1*, 31 for *PR1*, 33 for *PDF1.2A* and 29 for *ACTIN*. The primers used for sequence amplification were MyoB1-FP1 (5'-GGGTTCACGGAGTTTCACTAGAGC-3'), MyoB1-RP1 (5'-CAGCATTGGACTTATTGGTTGTAGC-3'), ACTIN-FP1 (5'-AT GAAGATTAAGGTCGTGGCA-3'), ACTIN-RP1 (5'-GTTTTTATCCGAGTTTGAA GA-3'), PR1-FP1 (5'-TTCCCTCGAAAGCTCAAGATA-3'), PR1-RP1 (5'-GGC ACATCCGAGTCTCACTGAC-3'), PDF1.2A-FP1 (5'-TAAGTTTGCTTCCATCATC ACCC-3') and PDF1.2A-RP1 (5'-GTGCTGGGAAGACATAGTTGC-3').

Generation of *pMyoB1:GUS* plant lines

The *pMyoB1:GUS* construct was generated by Gateway cloning (Life Technologies, Grand Island, NY, USA). The promoter region (*c*. 2 kb) of *MyoB1* comprised the genomic DNA sequence upstream of this gene and the start codon. This genomic region of Col-6 was amplified by PCR with the *attB* recombination site-containing primers MyoB1p-F (5'-GGGGACAAGT TTGTACAAAAAAGCAGGCTAATTTTATATACACAAATAAAGAAACCAGA-3') and MyoB1p-R (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCATCTGT AATTTTACTGCAGACG-3'). The PCR product was recombined into the donor vector pDONR-zeo (Life Technologies) to generate an entry clone. Sequencing of the cloned DNA stretch revealed that the sequences of *MyoB1* promoter regions in Col-0 and Col-6 are identical. The pMDC163 vector (Curtis and Grossniklaus, 2003), which carries a *GUS* reporter gene, served as the destination vector for the *MyoB1* promoter region present in the entry clone. The resulting *pMyoB1:GUS* construct was transformed into Col-6 by *Agrobacterium tumefaciens* (strain GV3101::pMP90)-mediated gene transfer (Clough and Bent, 1998).

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SUPPORTING INFORMATION

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

Fig. S1 Various Arabidopsis enhancer-trap β-glucuronidase (GUS) staining patterns were seen in the primary and secondary screens.

Fig. S2 T-DNA insertion loci diagrams for enhancer-trap lines A, I and J.

Fig. S3 Enhancer-trap line *myob1-2* exhibits various β-glucuronidase (GUS) staining patterns.

Fig. S4 Canonical plant defence pathway marker genes are not influenced directly by *MyoB1* (*Myosin Binding Protein 1*).

Fig. S5 *MyoB1* (*Myosin Binding Protein 1*) mutants show no change in response to *Pseudomonas syringae* bacterial infection relative to wild-type Col-0 plants. CFU, colony-forming unit; FW, fresh weight.

Fig. S6 Interrupted *MyoB1* (*Myosin Binding Protein 1*) paralog At1g74830 exhibits reduced susceptibility to *Hyaloperonospora arabidopsidis* (*Hpa*).

Table S1 Seven independent hyphae-associated response enhancer-trap lines.