The Lateral Organ Boundaries Domain Transcription Factor LBD20 Functions in Fusarium Wilt Susceptibility and Jasmonate Signaling in Arabidopsis[1][W]

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The *LATERAL ORGAN BOUNDARIES* (*LOB*) *DOMAIN* (*LBD*) gene family encodes plant-specific transcriptional regulators functioning in organ development. In a screen of Arabidopsis (*Arabidopsis thaliana*) sequence-indexed transferred DNA insertion mutants, we found disruption of the *LOB DOMAIN-CONTAINING PROTEIN20* (*LBD20*) gene led to increased resistance to the root-infecting vascular wilt pathogen *Fusarium oxysporum*. In wild-type plants, *LBD20* transcripts were barely detectable in leaves but abundant in roots, where they were further induced after *F. oxysporum* inoculation or methyl jasmonate treatment. Induction of *LBD20* expression in roots was abolished in *coronatine insensitive1* (*coi1*) and *myc2* (allelic to *jasmonate insensitive1*) mutants, suggesting LBD20 may function in jasmonate (JA) signaling. Consistent with this, expression of the JA-regulated *THIONIN2.1* (*Thi2.1*) and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) genes were up-regulated in shoots of *lbd20* following treatment of roots with *F. oxysporum* or methyl jasmonate. However, *PLANT DEFENSIN1.2* expression was unaltered, indicating a repressor role for LBD20 in a branch of the JA-signaling pathway. Plants overexpressing *LBD20* (*LBD20-OX*) had reduced *Thi2.1* and *VSP2* expression. There was a significant correlation between increased *LBD20* expression in the *LBD20-OX* lines with both *Thi2.1* and *VSP2* repression, and reduced survival following *F. oxysporum* infection. Chlorosis resulting from application of *F. oxysporum* culture filtrate was also reduced in *lbd20* leaves relative to the wild type. Taken together, LBD20 is a *F. oxysporum* susceptibility gene that appears to regulate components of JA signaling downstream of COI1 and MYC2 that are required for full elicitation of *F. oxysporum*- and JA-dependent responses. To our knowledge, this is the first demonstration of a role for a *LBD* gene family member in either biotic stress or JA signaling.

Plants have evolved inducible defense mechanisms to protect against microbial pathogens, and these include cell wall modifications, the production of antimicrobial metabolites and proteins, and in some instances, hypersensitivity via programmed cell death processes. Several of these host defense responses are transcriptionally regulated via the action of a suite of defense hormones in plants, including salicylic acid (SA), jasmonate (JA), and ethylene (Schenk et al., 2000; Pieterse et al., 2009). In turn, microbial pathogens have evolved mechanisms, such as secreted effector molecules, that avert the activation of these host defense responses (Jones and Dangl, 2006; Boller and He, 2009). Pathogens also reprogram host physiological functions to enhance susceptibility. For example, bacterial and fungal pathogens that enter leaves via stomatal apertures secrete molecules that block stomatal closure (Hok et al., 2010). A further extension of host reprogramming by pathogens is where host processes regulated by growth hormones such as auxins, gibberellins, and cytokinins, are modified by pathogens either by the production of functional hormone analogs by the pathogen itself or via modification of endogenous hormone levels (Robert-Seilaniantz et al., 2007; Grant and Jones, 2009). Alternatively, pathogens can intercept hormone-signaling processes to provide conditions more conducive for infection (Bari and Jones, 2009; Kazan and Manners, 2009). Therefore, to understand the contribution of the host to the final disease outcome, it is necessary to consider pathogen-targeted host processes that may enhance susceptibility in addition to the more commonly studied processes of pathogen perception and the activation of the host defense system.

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Fusarium oxysporum is a root-infecting fungal pathogen that causes wilt disease on a broad range of economically important plant species and also the model plant Arabidopsis (*Arabidopsis thaliana*; Dombrecht et al., 2006; van Hemelrijck et al., 2006; Berrocal-Lobo and Molina, 2008; Michielse and Rep, 2009). This pathogen is soilborne and enters the plant initially through the roots, and subsequently colonizes the vascular tissues and xylem vessels before moving up to stem and foliar tissues (Lagopodi et al., 2002; Czymmek et al., 2007). *F. oxysporum* is considered to be a hemibiotroph (Thaler et al., 2004) because the initial stages of root infection by this pathogen appear to be biotrophic (Czymmek et al., 2007), while later stages of the infection cycle, particularly the wilting and lesions that occur in foliar tissues, are more typical of the symptoms incited by necrotrophic pathogens. The genomes of pathogen strains of *F. oxysporum* infecting tomato (*Solanum lycopersicum*) and Arabidopsis have been sequenced (Ma et al., 2010; Thatcher et al., 2012). Infection by *F. oxysporum* involves secreted pathogen effector proteins, some of which are encoded on supernumerary pathogenicity chromosomes (Ma et al., 2010). These effectors act as either virulence or avirulence factors, depending on the host genotype (Rep and Kistler, 2010; Takken and Rep, 2010; Thatcher et al., 2012).

The ability to study *F. oxysporum* interactions on the model host Arabidopsis has opened up diverse genetic- and genomic-based approaches to identify and characterize host factors involved in Fusarium wilt disease development. For example, there is variation in the response to *F. oxysporum* across Arabidopsis ecotypes and the partial resistance of the commonly studied Columbia-0 (Col-0) ecotype is inherited as a quantitative trait (Diener and Ausubel, 2005). One quantitative trait locus contains the atypical resistance gene *RESISTANCE TO FUSARIUM OXYSPORUM1* that encodes WALL-ASSOCIATED KINASE-LIKE KINASE22, but how this gene contributes to resistance is currently unknown (Diener and Ausubel, 2005). One possibility discussed by Diener and Ausubel (2005) is that resistance may be at least partially mediated via SA-regulated defenses. Exogenously applied SA provides increased resistance to this pathogen (Edgar et al., 2006) and transgenic and mutant genotypes that are impaired in SA accumulation have enhanced susceptibility (Diener and Ausubel, 2005; Thatcher et al., 2009).

Recent studies indicate that successful *F. oxysporum* infection requires the action of diverse host hormonal-signaling pathways, their associated transcriptional regulators, and downstream-regulated response genes. In contrast to SA, some hormone-signaling pathways appear to promote disease susceptibility to *F. oxysporum* in Arabidopsis. Several components of Arabidopsis auxin-signaling pathways and polar auxin transport processes, but not auxin biosynthesis itself, have been shown to be required for full virulence of *F. oxysporum* on Arabidopsis (Kidd et al., 2011), suggesting a link between infection and development (Kazan and Manners, 2009). Application of abscisic acid (ABA), a plant hormone usually associated with abiotic stress responses such as water deficit, stimulated increased Fusarium wilt disease development in Arabidopsis while mutations in ABA biosynthesis genes promoted resistance (Anderson et al., 2004). This suggested that ABA signaling may act to prioritize abiotic stress tolerance processes over defense to pathogens like *F. oxysporum* (Anderson et al., 2004).

The role of the JA-signaling pathway in Arabidopsis-*F. oxysporum* interactions is of particular interest as it appears to have two opposing effects that either repress or stimulate disease development. The eventual disease outcome reflects the relative balance of these two JA-regulated processes (Thatcher et al., 2009). First, it appears that JA-regulated defenses contribute positively to resistance. For example, the JA-regulated *THIONIN2.1* (*Thi2.1*) gene, which encodes an antimicrobial thionin protein, inhibits infection by *F. oxysporum* when overexpressed in transgenic plants (Epple et al., 1997; Chan et al., 2005). Overexpression of transcriptional activators (e.g. ETHYLENE RESPONSE FACTOR1 [ERF1]) of JA-responsive defense genes also reduces *F. oxysporum* infection (Berrocal-Lobo et al., 2002; McGrath et al., 2005). On the other hand, negative transcriptional regulators of JA-responsive defense genes (e.g. ERF4 and MYC2) confer increased *F. oxysporum* susceptibility (Anderson et al., 2004; McGrath et al., 2005). JAs act in plants by their conjugated form being recognized by the CORONATINE INSENSITIVE1 (COI1) protein (Katsir et al., 2008), and surprisingly the Arabidopsis *coi1* mutant shows strong resistance to *F. oxysporum* despite greatly reduced JA-dependent defenses (Thatcher et al., 2009). Interestingly, *coi1* is also nonresponsive to chlorosis-inducing factors present in culture filtrates of *F. oxysporum* (Thatcher et al., 2009). The strong resistance to *F. oxysporum* observed in *coi1* mutants has been proposed to be due to a reduction of JA-induced senescence, which is exploited by the pathogen to cause disease symptoms such as chlorosis and necrosis at late stages of infection (Thatcher et al., 2009). Similarly analysis of the *constitutive expression of pr genes5/hypersenescence1* mutant with constitutively active defenses and enhanced senescence response, shows increased *F. oxysporum* susceptibility (Schenk et al., 2005). More recently, the MEDIATOR25/PHYTOCHROME AND FLOWERING TIME1 (MED25/PFT1) subunit of the plant mediator complex, which positively regulates the JA-responsive defense genes, has also been shown to act as a *F. oxysporum* susceptibility gene and mutants show reduced expression of JA-responsive genes but increased *F. oxysporum* resistance (Kidd et al., 2009).

Despite the importance of root pathogens in plant agriculture and natural ecosystems, much less is known about defense signaling by roots when compared with that of aerial plant organs (Okubara and Paulitz, 2005; Erb et al., 2009). Because *F. oxysporum* infects via the roots it would be expected that key signaling events

determining resistance and susceptibility are initiated by both host and pathogen in root tissues early on during infection. The JA-signaling pathway and downstream responses, but not those of the SA pathway, appear to be activated in both the roots and leaves of Arabidopsis during infection by *F. oxysporum* (Edgar et al., 2006; Thatcher et al., 2009; Kidd et al., 2011). Activation of JA-regulated genes in leaves was also observed within 24 h after inoculation (Kidd et al., 2011), prior to the invasion of foliar tissues by the fungus, suggesting that systemic signals of host and/or pathogen origin are most likely transmitted from root to shoot during *F. oxysporum* infection. The critical importance of JA signaling in infected roots was elegantly demonstrated using the *coi1* mutant in grafting experiments (Thatcher et al., 2009). It was found that plants consisting of a *coi1* rootstock with wild-type scion had strong resistance to *F. oxysporum* similar to that of plants with *coi1* rootstock and *coi1* scion. In contrast, plants with a wild-type rootstock and a *coi1* scion remained susceptible, indicating that JA-perception and -signaling processes in the roots are critical in determining the eventual disease outcome in Fusarium wilt disease. However, our understanding of the genes involved in JA signaling that determines resistance and susceptibility to *F. oxysporum* is very limited.

The aim of this study was to identify novel root-expressed genes of Arabidopsis that are required for susceptibility to *F. oxysporum*, and then to characterize these genes to determine whether they have roles in JA signaling and plant defense regulation or other mechanisms. The approach that we adopted was to initially undertake large-scale unbiased screening of a collection of defined sequence-indexed transferred DNA (T-DNA) insertion mutants of Arabidopsis (O'Malley and Ecker, 2010) and identify mutants that had a reproducible increase in resistance to infection by *F. oxysporum* when compared with that of the wild type. This was followed by verification of the observed resistant phenotype for the candidate gene by testing a second independent mutant carrying a distinct T-DNA insertion allele in the candidate gene. Because we were particularly interested in genes that function in roots, we then undertook expression analysis of the candidate genes in wild-type plants to test for root expression. In this article, we report on the *LATERAL ORGAN BOUNDARIES* (*LOB*) *DOMAIN-CONTAINING PROTEIN20* (*LBD20*) gene that was identified through this process. *LBD20* is a member of the plant-specific *LBD* gene family and we present evidence herein that LBD20 has a novel role as a predominantly root-expressed negative regulator of both resistance to *F. oxysporum* and a subset of JA responses. The LBD family has previously mainly been studied in regard to plant development with roles in defining the boundaries between organs (Shuai et al., 2002; Majer and Hochholdinger, 2011; Feng et al., 2012). Other LBDs have been shown to have functions in the regulation of nitrogen metabolism and anthocyanin biosynthesis (Rubin et al., 2009). Functions of LBD20 were previously unknown, and our results demonstrate LBD20 is the first member of the LBD family shown to have a role in JA signaling and plant-pathogen interactions.

RESULTS

Large-Scale Screening of Arabidopsis Mutants Identifies *LBD20* as an *F. oxysporum* Susceptibility Gene

To identify novel genes that affect resistance and susceptibility to *F. oxysporum*, we systematically screened an Arabidopsis sequence-indexed T-DNA insertion mutant collection (CS27941) consisting of 6,868 T-DNA insertion lines for an altered disease phenotype when compared with that of the wild-type Col-0. Disease phenotypes were determined at 7 and 14 d post inoculation by recording the percentage of diseased plants, survival ratio, and a disease score (rated on a 0–5 scale with 0 being highly resistant and 5 being highly susceptible; see Supplemental Fig. S1). Mutants that showed statistically significant ($P < 0.01$) disease development compared with Col-0 were selected and rescreened for confirmation of a significantly altered disease phenotype. One of the mutants recovered from this process that showed increased resistance was SALK_020410C (designated here as *lbd20-1*) and has a T-DNA insertion in the intron of the *LBD20* gene. A second homozygous independent mutant line of *LBD20* designated as *lbd20-2* was obtained (SALK_054710C) with a T-DNA insertion in exon 1 (Fig. 1A). Both *lbd20* mutants were confirmed by quantitative reverse transcription (qRT)-PCR to be similarly compromised for *LBD20* transcript levels when compared with that of the wild type and are thus expected to be nonfunctional (Supplemental Fig. S2A). Both *lbd20* mutants showed significantly increased resistance to *F. oxysporum* when compared with the wild type both in disease symptoms and plant survival (Fig. 1, B–D). This further indicated a role for LBD20 in susceptibility to Fusarium wilt disease and represents the first case of a *LBD* gene being implicated in resistance or susceptibility to any plant pathogen, to our knowledge.

LBD20 Is Preferentially Expressed in Roots and Responsive to *F. oxysporum* Infection

As stated above, we were particularly interested in genes expressed within root tissues where *F. oxysporum* penetration and the early stages of infection take place, and several *LBD* genes have previously been shown to be expressed in roots (Shuai et al., 2002; Feng et al., 2012). To test this in further detail for the *LBD20* gene, we monitored its expression in wild-type shoot and root tissues before and after *F. oxysporum* inoculation (Fig. 2). *LBD20* expression in shoots could not be reliably detected using qRT-PCR, suggesting it is either very lowly expressed in shoot tissues or expressed

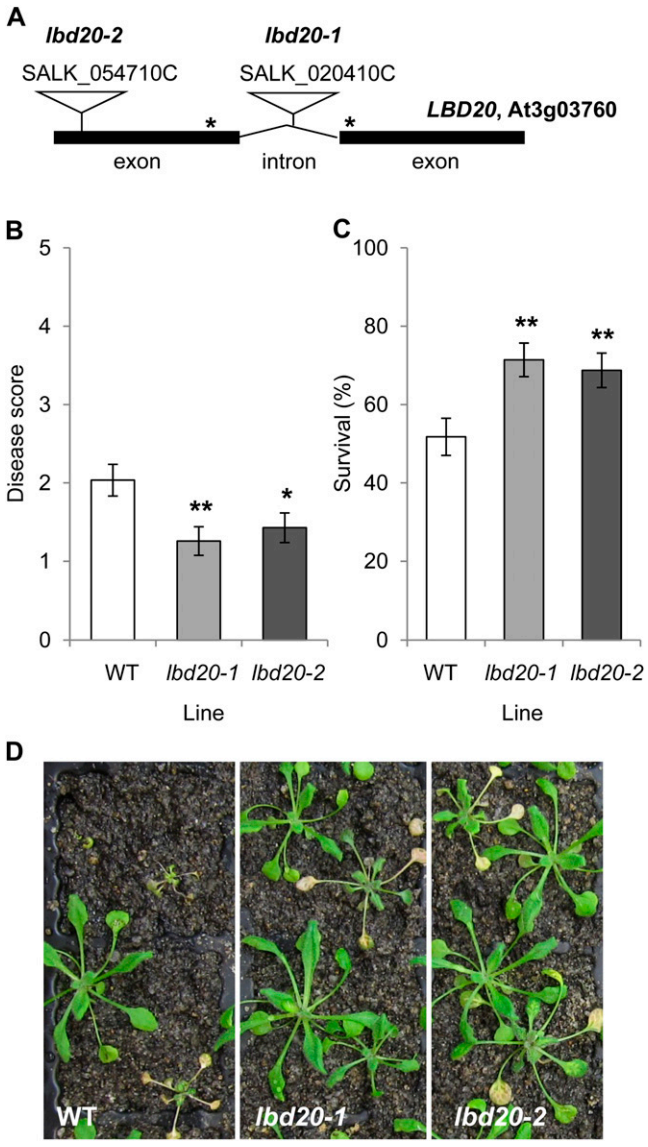

Figure 1. Two independent *lbd20* T-DNA mutant lines exhibit increased resistance to *F. oxysporum*. A, Schematic representation of *lbd20-1* and *lbd20-2* T-DNA insertion lines. Not drawn to scale. Primer binding sites for determination of *LBD20* expression are noted with asterisks. B to D, Disease phenotypes of *F. oxysporum* inoculated plants with disease score at 14 d post inoculation (B), survival at 21 d post inoculation (C), and representative images of plants 14 d post inoculation (D). The average of three biological replicates consisting of 14 plants each is shown with SE. Asterisks indicate values that are significantly different (**$P < 0.01$, *$P < 0.05$ Student's *t* test) from the wild type (WT).

within very specific shoot cell types. We did, however, readily measure *LBD20* expression in roots just prior to inoculation (time point 0) and after inoculation (Fig. 2). *LBD20* expression in 3-h mock-treated samples also increased 2-fold over time point 0 samples, suggesting *LBD20* expression is responsive to the inoculation method that involved the potential wounding of roots as they are removed from soil, dipped in water (mock treatment), and repotted. *LBD20* expression in *F. oxysporum*-inoculated samples was significantly higher than those of mock treatments at both 3 and 24 h (Fig. 2). Combined, these results indicate *LBD20* is predominantly root expressed and responsive to *F. oxysporum* infection.

LBD20 Expression Is COI1 and JA Regulated

We previously determined that the JA receptor COI1 plays a vital role in susceptibility to *F. oxysporum*, in particular in root tissues where wild-type scions grafted onto rootstocks in which the *COI1* gene has been silenced show complete resistance to *F. oxysporum* disease symptom development (Thatcher et al., 2009). To determine if LBD20 functions within the framework of the COI1-dependent JA-signaling pathway, we monitored *LBD20* expression in wild-type and *coi1* roots following *F. oxysporum* inoculation and found the induced expression of *LBD20* to be completely abolished in *coi1* (Fig. 3A). This prompted us to examine the potential JA inducibility of *LBD20*. Expression of *LBD20* was induced in root tissues following the transfer of seedlings to methyl jasmonate (MeJA)-containing growth medium (3.8-fold over mock) and this induction was also COI1 dependent (Fig. 3B). These results demonstrate that *LBD20* is regulated by the COI1-dependent signaling pathway and response.

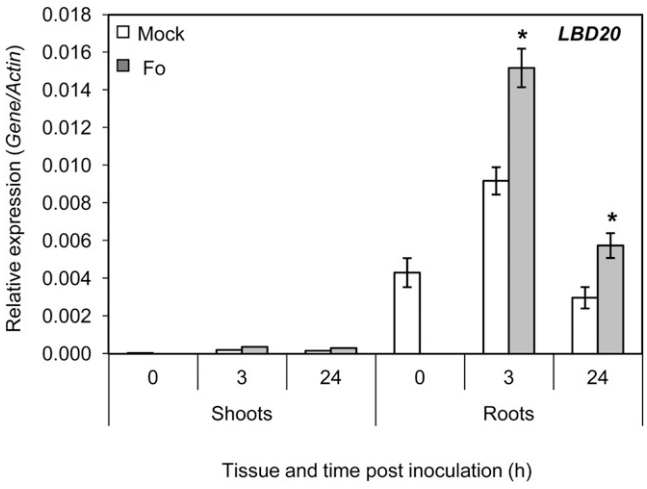

Figure 2. *LBD20* is root expressed and responsive to *F. oxysporum* infection. *LBD20* expression was monitored in wild-type (WT) shoot and root tissue at time 0 h (no treatment) and in mock or *F. oxysporum* (Fo) challenged plants at 3 and 24 h post inoculation. 0 represents roots and shoots taken from plants just prior to inoculation. The average of three biological replicates consisting of pools of 20 to 30 plants is shown with SE. Gene expression levels are relative to the internal control *β-actin* genes. Asterisks indicate values that are significantly different (*$P < 0.05$ Student's *t* test) from mock treatment at the same time point.

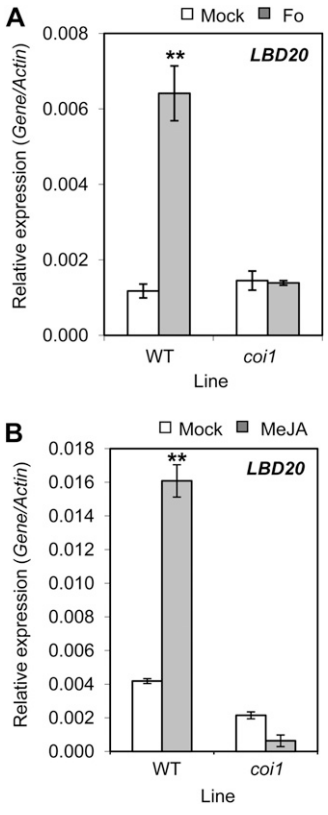

Figure 3. *F. oxysporum*- and JA-induced *LBD20* expression is COI1 dependent. *LBD20* expression was monitored in root tissue of mock or *F. oxysporum* (Fo) challenged wild-type (WT) or *coi1* plants at 96 h post infection (A), and in WT and *coi1* plants 6 h post mock or MeJA treatment (B). The average of three biological replicates consisting of pools of 10 to 30 plants is shown with SE. Gene expression levels are relative to the internal control *β-actin* genes. Asterisks indicate values that are significantly different (**$P < 0.01$ Student's *t* test) from mock treatment within the same line. Similar results were obtained in an independent experiment.

MeJA-Induced Expression of *LBD20* Is Dependent on MYC2

One of the key regulatory genes in early JA-signaling events downstream of COI1 is the transcriptional regulator MYC2 that is thought to act, at least in part, by binding to the G-box cis-element (5′-CACGTG-3′). The *LBD20* promoter contains two potential MYC2-binding G-box motifs (−794 to −789, and −338 to −333, relative to the predicted transcription start site). To test whether MYC2 regulates *LBD20*, we examined *LBD20* expression within the *myc2* mutant background and found MeJA-induced expression of *LBD20* in roots was abolished in *myc2* (Fig. 4A). We also examined *LBD20* in MYC2-overexpressing (*MYC2-OX*) plants (*35S::MYC2*) without providing any MeJA stimulus (Fig. 4, B and C). *LBD20* expression, however, did not differ between wild-type and *MYC2-OX* plants. These findings suggest that LBD20 is part of the JA and MYC2 transcriptional regulon but that up-regulation of *MYC2* expression alone is insufficient to stimulate expression of *LBD20*. Further investigations are required to determine whether these potential MYC2-binding motifs found in the *LBD20* promoter are functional and/or positive. Regulation of *LBD20* by MYC2 may require JA or pathogen treatment. We also examined *MYC2* expression in the *lbd20* mutant background and found no difference in *F. oxysporum* or MeJA induction patterns compared with wild-type plants in either shoot or root tissues (data not shown).

LBD20 Is a Repressor of a Subset of JA-Regulated Defense Genes in Shoot Tissues

The up-regulation of *LBD20* in *F. oxysporum*- and MeJA-treated wild-type plants and its COI1 and MYC2 dependency prompted us to examine the expression of four well-established marker genes (*Thi2.1*, *VEGETATIVE STORAGE PROTEIN2* [*VSP2*], *PLANT DEFENSIN1.2* [*PDF1.2*], and *PATHOGENESIS RELATED4* [*PR4*]) for downstream JA-regulated defense responses in the *lbd20* mutant. Initially, we examined root tissue following treatment with MeJA. Although transcripts for only *Thi2.1*, *VSP2*, and *PDF1.2* were induced in root tissue, there was no apparent difference in the induction between the *lbd20* mutant and the wild type (Fig. 5A; Supplemental Fig. S3A). However, examination of shoot tissue indicated that all four genes were MeJA induced and that a subset of these JA-response genes were differentially regulated in the *lbd20* mutant. For example, expression of the *Thi2.1* and *VSP2* genes were more strongly induced ($P < 0.05$) in the *lbd20* mutant than the similarly treated wild type (Fig. 5B). In a separate confirmatory experiment, the repressive function on defense gene expression was also confirmed for both *lbd20* alleles (e.g. Supplemental Fig. S2B). In contrast, there was no difference detected in the expression of the *PDF1.2* and *PR4* genes in shoots following MeJA treatment (Supplemental Fig. S3B). We also examined *Thi2.1* and *VSP2* expression following *F. oxysporum* inoculation and found the same induction pattern in shoots and roots (Supplemental Fig. S4). These results suggest that LBD20 plays a role in JA signaling and acts as a repressor of a subgroup of JA- and pathogen-induced defense genes in shoots. Given the predominant root expression of the *LBD20* gene (Fig. 2), it is possible that LBD20 may either function in a specialized JA-related root-to-shoot signaling process or that very low expression levels in shoots may be sufficient for this regulatory role.

F. oxysporum Culture Filtrate-Induced Chlorosis Is Reduced in *lbd20*

We have previously observed that factors present in *F. oxysporum* culture filtrates induce a senescence-like chlorotic phenotype in wild-type leaves and this response is absent in the *coi1* mutant, which is also highly resistant to *F. oxysporum* disease symptom development

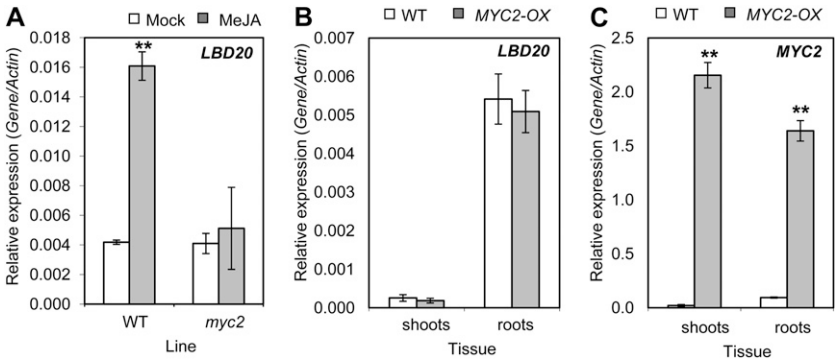

Figure 4. *LBD20* MeJA-induced expression is MYC2 dependent. A, *LBD20* expression was examined in wild-type (WT) and *myc2* root tissue 6 h post mock or MeJA treatment. B and C, *LBD20* and *MYC2* expression were examined in shoot and root tissue of WT and *35S::MYC2* (*MYC2-OX*) plants. *MYC2* was examined to confirm its overexpression. Gene expression levels are relative to the internal control *β-actin* genes. The average of three biological replicates consisting of pools of 30 plants is shown with SE. Asterisks indicate values that are significantly different (**$P < 0.01$ Student's *t* test) from mock treatment within the same line (A) or WT within the same tissue (B and C). Similar results were obtained in an independent experiment.

(Thatcher et al., 2009). To determine if the increased *F. oxysporum* resistance in *lbd20* also affects this phenotype, we applied *F. oxysporum* culture filtrate to wild-type and *lbd20* detached leaves, alongside *coi1* and *myc2* (Fig. 6). We included *myc2*, as this mutant also exhibits increased resistance to *F. oxysporum* (Anderson et al., 2004) and regulates *LBD20* (Fig. 4), but its response to *F. oxysporum* culture filtrate was unknown. All three of the mutants tested had reduced lesion development compared with wild-type leaves. Interestingly, the *coi1* mutant was the most insensitive to this treatment, with *myc2* and *lbd20* mutants showing intermediate sensitivity compared with the wild type. Although the secreted *F. oxysporum* elicitors that may induce the chlorotic phenotype are currently unknown, the nonresponsiveness and reduced responsiveness of *coi1* and *myc2* leaves, respectively, to *F. oxysporum* culture filtrate, suggest that possible fungal elicitors act through the JA-signaling pathway. Combined, these results imply LBD20 may also contribute to a JA-signaling-dependent host sensitivity to fungal elicitors of host senescence and chlorosis.

Increased *LBD20* Expression Correlates with Reduced *Thi2.1* and *VSP2* Expression and Susceptibility to *F. oxysporum*

To further characterize the role of LBD20 in defense and JA signaling, we generated *LBD20*-overexpressing plants (*LBD20-OX*). We noted that the *LBD20-OX* plants suffered from varying degrees of lobed leaves, sterility, and termination of development (Supplemental Fig. S5A). Five of the 24 recovered *LBD20-OX* plants appeared similar to the wild type, nine were sterile, and five died. Similar phenotypes have been observed in plants overexpressing some other *LBD* genes (Shuai et al., 2002; Nakazawa et al., 2003; Naito et al., 2007). *LBD20-OX* transformants with milder phenotypes set viable seed and were used in subsequent experiments. From these T2 plants, two lines in either the wild-type Col-0 background or the *lbd20* background, and carrying only one *LBD20-OX* insertion, were analyzed for *LBD20* expression (Fig. 7A).

To test the hypothesis that LBD20 is a repressor of a subset of JA-regulated defense genes, we analyzed the

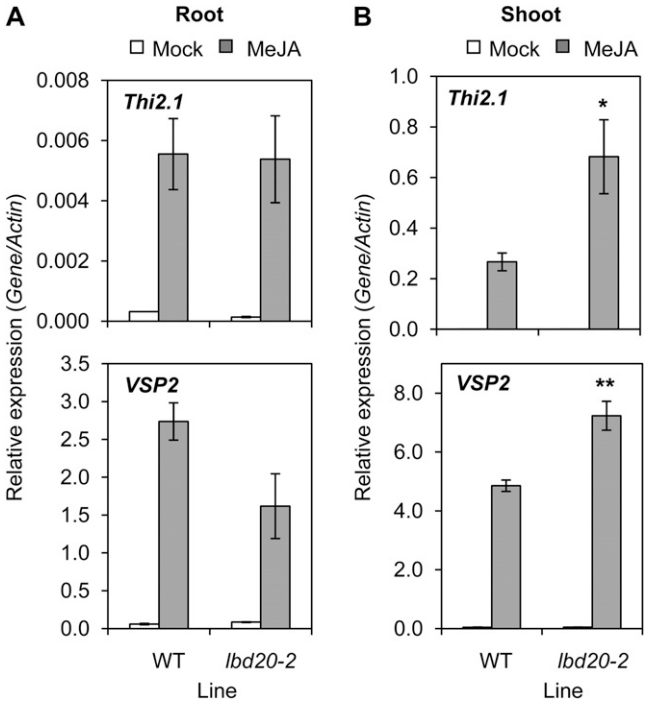

Figure 5. LBD20 is a repressor of a subset of JA-regulated defense genes. Expression of JA-response genes was examined in wild-type (WT) and *lbd20* root (A) or shoot tissue (B) 6 h post mock or MeJA treatment. Gene expression levels are relative to the internal control *β-actin* genes. The average of three biological replicates consisting of pools of 30 to 40 plants is shown with SE. Asterisks indicate values that are significantly different (**$P < 0.01$, *$P < 0.05$ Student's *t* test) from WT. Similar results were obtained in independent experiments.

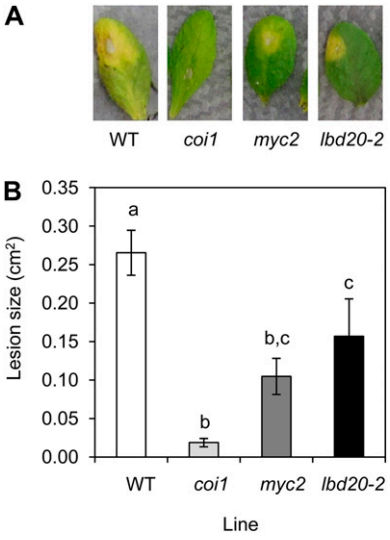

Figure 6. *coi1*, *myc2*, and *lbd20* leaves are less sensitive to *F. oxysporum* culture filtrate-induced lesions. A, *F. oxysporum* culture filtrate initiates a senescence response in wild-type (WT) leaves but lesion size is reduced in *coi1*, *myc2*, and *lbd20*. Representative leaves are shown at 3 d post treatment. Mock treatments of potato dextrose broth (PDB) and water showed no phenotype (data not shown). B, Average lesion size from 15 leaves shown with SE. Letters indicate values that are significantly different from each other ($P < 0.05$, all pairs Student's *t* test). Similar results were obtained in an independent experiment.

expression of *Thi2.1*, *VSP2*, and *PDF1.2* in wild-type and *LBD20-OX* plants after mock or MeJA treatment. Following MeJA treatment, three of the four *LBD20-OX* lines exhibited significantly ($P < 0.01$) lower expression of both *Thi2.1* and *VSP2* than wild-type plants, while *LBD20-OX-2* only had significantly reduced *Thi2.1* expression ($P < 0.05$; Fig. 7, B and C). Of the four *LBD20-OX* lines tested, *LBD20-OX-2* also had the lowest *LBD20* levels (Fig. 7A) and exhibited no altered leaf morphology (Supplemental Fig. S5B). This suggests a threshold level of *LBD20* expression may be required to observe its effects on plant development. Indeed, we found a strong negative correlation between *LBD20* levels and MeJA inducibility of *Thi2.1* and *VSP2* (Fig. 7D). Consistent with the hypothesis that LBD20 only represses a subset of JA-regulated defense genes, we found no correlation between *PDF1.2* and *LBD20* expression in the *LBD20-OX* lines (Fig. 7D).

We also inoculated wild-type plants and *LBD20-OX* lines with *F. oxysporum* and monitored disease symptom development. There were also strong correlations between both the levels of basal ($R^2 = -0.77$) and JA-induced ($R^2 = -0.80$) *LBD20* expression across the *LBD20-OX* lines and reduced plant survival following inoculation with *F. oxysporum* (Fig. 8). The diminishing survival of inoculated plants with increasing *LBD20* expression strongly supports the conclusion that *LBD20* acts as a *F. oxysporum* susceptibility gene in Arabidopsis.

DISCUSSION

We initially identified *LBD20* as a host-susceptibility gene from a screen of nearly 7,000 independent homozygous sequence-indexed T-DNA insertion lines for increased resistance to the root-infecting pathogen *F. oxysporum*. Among the mutants identified, *lbd20* was selected for further analysis because its increased resistance was confirmed in a second independent homozygous T-DNA insertion line, and because *LBD20* was predominantly expressed in roots, the site of primary infection. Even though *F. oxysporum* infects and colonizes through root tissues, our understanding of host gene functions in root tissue during infection is very limited. LBD20 belongs to the plant-specific LBD family consisting of 42 members in Arabidopsis based on the defining member LOB (Shuai et al., 2002). *LOB* was initially identified from an enhancer trap screen for genes expressed at the adaxial base of initiating lateral organs and other family members identified based on conservation of the so-called N-terminal LOB domain (Shuai et al., 2002). For most LBD family members the function is not known, but for some, roles have been defined in organ development, anthocyanin, and nitrogen metabolism, as well as in responses to phytohormones such as cytokinin, auxin, and gibberellin (Borghi et al., 2007; Bureau et al., 2010; Majer and Hochholdinger, 2011; Feng et al., 2012). To our knowledge, our findings represent the first functional characterization of LBD20 and the first demonstration of a role for any LBD family member in plant-pathogen interactions.

The JA-signaling pathway appears to play two contrasting roles in plant responses to *F. oxysporum* infection (Thatcher et al., 2009). One JA-regulated pathway promotes susceptibility via the pathogen hijacking JA-induced senescence processes for disease symptom development. Another JA-regulated pathway appears to promote resistance via the expression of JA-induced defenses such as antifungal proteins like thionins, defensins, and chitinases. While it is known that defense gene overexpression or up-regulation in shoot tissues is associated with increased resistance (Epple et al., 1997; Tierens et al., 2002; Anderson et al., 2004; Berrocal-Lobo and Molina, 2004; McGrath et al., 2005; van Hemelrijck et al., 2006), it is not the dominant JA-regulated process in determining the disease outcome during *F. oxysporum* infection because mutants compromised in defense gene expression such as *coi1* and *pft1* are still highly resistant to this pathogen (Kidd et al., 2009; Thatcher et al., 2009). To determine the resistance mechanism in the *lbd20* mutants, we analyzed JA-mediated defense gene expression following *F. oxysporum* inoculation. A subset of JA-mediated defense genes were up-regulated following fungal infection in *lbd20* shoot tissues compared with wild-type plants, with the same pattern also observed after MeJA treatment (Fig. 5). This defense gene subset included that encoding the antifungal protein Thi2.1 known to reduce disease severity to

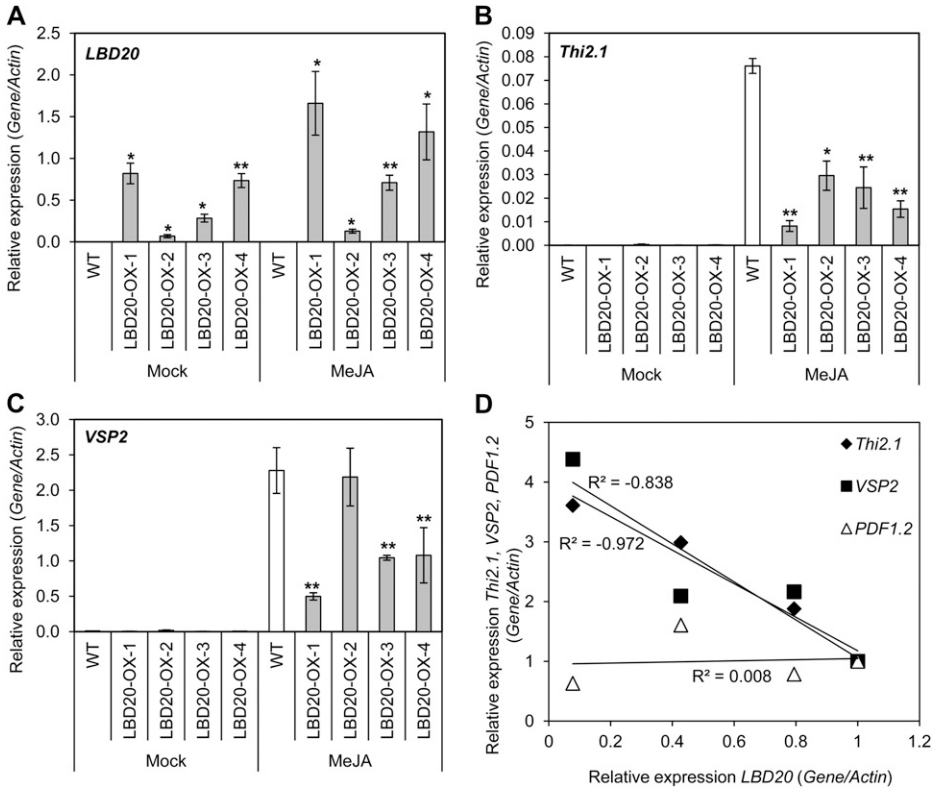

Figure 7. LBD20 is a repressor of a subset of JA-regulated defense genes. A to C, *LBD20*, *Thi2.1*, and *VSP2* expression was examined in shoot tissue of wild-type (WT) and *LBD20-OX* lines 6 h post mock or MeJA treatment. *LBD20-OX* lines 1 and 2 are in the WT background, while lines 3 and 4 are in the *lbd20-2* mutant background. Gene expression levels are relative to the internal control *β-actin* genes. The average of three biological replicates consisting of pools of 30 plants is shown with SE. Asterisks indicate values that are significantly different (**$P < 0.01$, *$P < 0.05$ Student's *t* test) from WT under the same treatment. No significant differences in expression levels among mock-treated samples were observed for *Thi2.1* or *VSP2*. D, MeJA-induced expression of defense marker genes in *LBD20-OX* lines was plotted against their internal *LBD20* expression. Displayed are trend lines.

F. oxysporum (Epple et al., 1997, 1998) and the anti-insect and wound-responsive protein VSP2, while no regulatory effect was observed on the plant defensin marker gene *PDF1.2*. The chlorosis and senescence-like response elicited by application of *F. oxysporum* culture filtrate was also reduced in *lbd20* (Fig. 6), although not to the degree seen in *coi1* that is insensitive to these *F. oxysporum* elicitors. These results suggest that the increased resistance observed in the *lbd20* mutants may be due to a combination of the enhanced production of some JA-regulated antifungal defense proteins such as thionins, as well as a partial reduction in JA-induced chlorosis and senescence processes that are required for symptom development.

The increased expression of some JA-regulated defense genes in the *lbd20* mutants indicates that LBD20 has a repressive role for a part of the JA plant-signaling pathway. It is possible that this may be mediated by transcriptional regulation of *LBD20* itself as it is also a JA- and *F. oxysporum*-responsive gene and its transcriptional induction by the fungus and JA is dependent on COI1 and MYC2, the respective JA receptor and a primary transcriptional regulator of JA signaling, respectively. It has been shown that a complex network of transcription factors are regulated downstream of MYC2 and suggested that different branches of this network are responsible for diverse JA-regulated functions (Dombrecht et al., 2007). This model is consistent with the notion that LBD20 regulates a component of JA signaling downstream of COI1 and MYC2. An analysis of public array data (Zimmermann et al., 2004; L. Thatcher, unpublished data) also identified altered JA-defense gene expression in the *lbd38* mutant with *PDF1.2* and *VSP2* expression severalfold higher in *lbd38* compared with wild-type plants, suggesting other LBD proteins may act as repressors of JA responses and this warrants a LBD family-wide investigation for this role.

Although it appears that the induction of *LBD20* by *F. oxysporum* infection and JA treatments requires MYC2, regulation downstream of LBD20 differs from that of MYC2. For example, *VSP2* induction was attenuated in *myc2* following MeJA treatment, while the defensin *PDF1.2*, *PR4* (Hevein-like, encoding a chitin-binding protein), and *Thi2.1* were up-regulated (Anderson et al., 2004; Lorenzo et al., 2004; L. Thatcher, unpublished data). Neither *PDF1.2* nor *PR4* expression was altered in *lbd20* compared with the wild type; however, both *Thi2.1* and *VSP2* were up-regulated. The MYC2 network (Dombrecht et al., 2007) may have repressive and activating branches for genes such as *PDF1.2* and *VSP2*, respectively. In this scenario, LBD20 may occupy a repressive branch or feedback loop that dampens *VSP2* expression. The MYC2 regulon is also differentially regulated in shoots and roots temporally during *F. oxysporum* infection (Anderson et al., 2004; Thatcher et al., 2009; L. Thatcher, unpublished data). In combination with upstream regulators, such as JASMONATE-ZIM-DOMAIN proteins, and downstream regulators, such as LBD20 and ERF1, the JA-dependent response can be finely tuned.

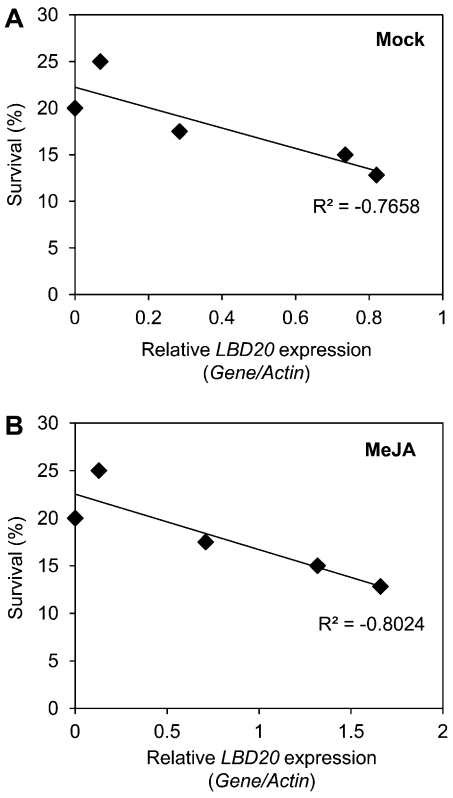

Figure 8. Increased *LBD20* expression correlates with increased susceptibility to *F. oxysporum*. Percentage survival of *F. oxysporum* inoculated wild-type and *LBD20-OX* plants plotted against their internal *LBD20* expression under mock (A) and JA-induced conditions (B). Displayed are trend lines. For *F. oxysporum* inoculation, the average of two biological replicates consisting of 20 plants each is shown.

In agreement with published data (Shuai et al., 2002), we found *LBD20* expression was virtually absent from leaves, but readily detected in roots (Fig. 2) and floral tissues (data not shown). The highly root-abundant expression of *LBD20* compared with that of shoots, taken in combination with the differential expression of JA-regulated defense genes in shoots of the *lbd20* mutants, suggests that either LBD20 has a role in a root-to-shoot signaling process that affects specific JA-responsive genes, or that very low levels of transcription of *LBD20* in shoots or in specific shoot cells are sufficient for its regulatory activity. Distinguishing these possibilities will require substantially more research, but there are several precedents for the systemic regulation of JA responses from remote tissues. Root inoculation of soybean (*Glycine max*) with the symbiont *Bradyrhizobium japonicum* led to activation of JA responses in shoots but not in roots (Kinkema and Gresshoff, 2008), and colonization of roots by certain soil bacteria, in particular *Pseudomonas* spp. and *Bacillus* spp., can protect aboveground plant tissues in a JA-dependent manner against different types of pathogens in a process known as induced systemic resistance (for review, see Van der Ent et al., 2009). Treatment with endogenous elicitors or wounding of roots also causes similar systemic effects. For example, wounding or systemin treatment of tomato roots caused induction of systemic responses in leaves and this was dependent on JA signaling (Li et al., 2002), and wounding of Arabidopsis roots caused increased JA biosynthesis in shoots but not roots (Hasegawa et al., 2011). Thus it is possible that LBD20 may be involved in the development of regulatory signals that move from roots to shoots.

Five other *LBD* genes display similar or root-specific expression patterns like *LBD20*, with two of these, LBD14 and LBD33, the closest phylogenetically related to LBD20 (Shuai et al., 2002; Matsumura et al., 2009). This suggests subgroups of LBD proteins may have unique roles in roots. In root tissues, the founding member *LOB* is expressed at the base of lateral roots and at the junction between the primary root and lateral root primordia (Shuai et al., 2002). These points are the preferential site of *F. oxysporum* colonization and penetration in Arabidopsis (Czymmek et al., 2007; Kidd et al., 2011). Although the cell-specific expression pattern of *LBD20* is unknown, it is tempting to speculate that specific root cells with increased levels of LBD20 are more susceptible to *F. oxysporum* infection. Examination of public array data for *LBD* genes (26 members with probe sets) responsive to MeJA or other necrotrophic pathogens indicates *LBD37* and *LBD41* are induced or repressed, respectively, >2-fold in response to *Alternaria brassicicola* and *Botrytis cinerea*. This implies other LBD proteins may function in disease responses to fungal pathogens.

LBD function is believed to be mediated, at least in part, through the LOB domain. This domain also determines the unique role of each LBD protein with LOB domains from other members unable to functionally replace each other (Matsumura et al., 2009; Majer and Hochholdinger, 2011). The LOB domain contains a Cys repeat (C motif), a conserved Gly residue, and a Leu-zipper-like motif. The C motif containing four cysteines is conserved in all LBD proteins and is predicted to form a DNA-binding zinc finger, while the less-conserved Leu-zipper-like motif is predicted to form a coiled-coil motif involved in homodimerization or other protein-protein interactions (Shuai et al., 2002; Majer and Hochholdinger, 2011). Nuclear localization and DNA-binding activity shown for some members suggests LBD proteins function as transcription factors (Shuai et al., 2002; Husbands et al., 2007; Naito et al., 2007; Rubin et al., 2009). Indeed, the LOB domain is sufficient for DNA-binding activity, and Husbands et al. (2007) found several LBD members could bind a 6-bp consensus motif (G)CGGC(G), termed the LBD motif, with a broader sequence of A/T C/T GCGGCG C/T/G A/G A/T. Direct promoter targets of LBD transcription factors are not yet known, though the extended LBD motif is present in a diverse set of over 40 genes (L. Thatcher, unpublished data). We found putative LBD motifs in the −1,000-bp *Thi2.1* (CTACGGCACTT) and *VSP2* promoters (GCACGGCTATG; GTGCGGCGAAT), but not in the *PDF1.2* promoter,

suggesting LBD20 may directly bind to the *Thi2.1* and *VSP2* promoters. Further experimental work will be required to confirm this. We also found no significant difference in *Thi2.1* or *VSP2* expression between root tissue of wild-type and *lbd20* plants, suggesting LBD20 either does not bind to these promoters or that other root- and shoot-specific transcription factors are required to mediate their tissue-specific expression.

While knockout mutations of most *LBD* genes show no obvious phenotypes, their overexpression in many cases results in leaf phenotypes like lobed and curled leaves, along with dwarfing and degrees of infertility (Shuai et al., 2002; Nakazawa et al., 2003; Naito et al., 2007; Mangeon et al., 2011). Similarly, we observed no obvious morphological changes in leaf or root morphology in either *lbd20* mutant, however both lines exhibited increased resistance to *F. oxysporum* accompanied by increased expression of a subset of JA-regulated defense genes. In affirmation, *LBD20-OX* plants had reduced JA-mediated defense gene expression (Fig. 7), but also suffered from varying degrees of altered leaf morphology, sterility, and development (Supplemental Fig. S5). Overexpression lines with minimal, or no abnormalities were selected for inoculation experiments, and it was shown that a correlation existed between *LBD20* expression, reduced *Thi2.1* and *VSP2* expression, and increasing plant susceptibility, measured as plant survival following inoculation (Figs. 7 and 8). Thus, results from the T-DNA insertion mutants and the overexpression transgenic plants support the notion that LBD20 contributes to susceptibility to *F. oxysporum*.

Another transcription factor that represses JA responses and functions within the LBD family framework is the MYB transcription factor MYB91/ASYMMETRIC LEAVES1 (AS1; Nurmberg et al., 2007). MYB91/AS1 binds to LBD6/AS2 to repress the expression of class 1 *KNOTTED-like homeobox* genes (for review, see Moon and Hake, 2011). In *as1/myb91* or *lbd6/as2* mutants the ectopic misexpression of *KNOTTED-like homeobox* genes results in plants with strongly lobed leaves. MYB91/AS1 also acts as a negative regulator of JA-inducible genes such as *Thi2.1* and *VSP1*, and as a susceptibility gene to the fungal necrotrophic pathogens *B. cinerea* and *A. brassicicola* (Nurmberg et al., 2007). Nurmberg and colleagues (2007) also showed MYB91/AS1 could bind to the promoters of over 30 genes responsive to *B. cinerea*, JA, or ethylene, including many involved in defense responses (e.g. *ERF*, *NUCLEOTIDE BINDING SITE-LEUCINE RICH REPEAT*, *GLUTATHIONE S-TRANSFERASE TAU7*, *CONSTITUTIVE EXPRESSION OF VSP1*, *Thaumatin* family gene). While MYB91/AS1-LBD6/AS2 binding implies LBD6 may be involved in defense responses, increased fungal resistance and up-regulation of JA defenses is not evident in *lbd6/as2* (Nurmberg et al., 2007). Some pathogens also actively target AS1 to induce disease symptom development. The tomato yellow leaf curl China virus effector βC1 competes with LBD6/AS2 for AS1 binding to selectively repress JA-responsive defense genes including *PDF1.2*, *PR4*, and *VSP1* (Yang et al., 2008). The *F. oxysporum* effector Fo5176-SECRETED IN XYLEM4 also promotes increased disease symptom development through a mechanism yet to be discovered, but it is suggested it may act with other Fo5176 effectors to activate components of the JA-signaling pathway (Thatcher et al., 2012). Other isolates of *F. oxysporum* and *Pseudomonas syringae* seem to target host JA signaling by secreting oxylipins or coronatine that mimic the host's endogenous JA signal (Miersch et al., 1999; Kloek et al., 2001; Katsir et al., 2008; Thatcher et al., 2009). These studies detail a common theme where pathogens selectively target host susceptibility genes to cause disease.

CONCLUSION

In summary, we identified *LBD20* as a novel highly root-expressed negative regulator of a subset of JA responses and as a susceptibility gene for Fusarium wilt disease. It will now be interesting to determine the role of other LBD family members in plant-pathogen interactions, to determine the active targets of LBD proteins, and to determine cell- and tissue-specific *LBD20* expression and function to explore the potential of LBD20 to regulate root-to-shoot signaling processes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Unless otherwise specified, all experiments were conducted with the wild-type Arabidopsis (*Arabidopsis thaliana*) Col-0 ecotype grown in soil under a short-day light regime (8-h ligh/16-h dark) at 21°C as described previously (Campbell et al., 2003; Edgar et al., 2006). For growth of seedlings on Murashige and Skoog salt plates, seeds were surface sterilized and plated on one-half-strength Murashige and Skoog (supplied with 3% Suc, 0.8% Bacto Agar, pH 7.2), stratified at 4°C, and incubated under the same conditions as soil-grown plants. The T-DNA insertion mutants (Alonso et al., 2003) *lbd20-1* (SALK_020410C), *lbd20-2* (SALK_054710C), *myc2* (SALK_061267C), and *coi1* (SALK_035548) were obtained from the Arabidopsis Biological Resource Centre. T-DNA mutants were confirmed for correct loci insert and homozygous state using the iSct Primers tool at http://signal.salk.edu/cgi-bin/tdnaexpress. *lbd20-1* plants were used for initial gene expression experiments under *F. oxysporum* infection. For all other experiments, the *lbd20-2* line was used. Homozygous *coi1* plants were selected on Murashige and Skoog plates containing 50 μM MeJA. *35S::MYC2* plants are described in Dombrecht et al. (2007). For generation of plants expressing the *LBD20* (*35S::LBD20*), the *LBD20* CDS was amplified using *LBD20-Hin*dIII-F 5′-GTTT-AAGCTTAACAATGGCTGATCAGCAGCGAG-3′ that includes an ATG start codon, and *LBD20-Eco*RI-R 5′-GGTAGAATTCTCATCTCCGGTGAAAATCC-3′. The resulting amplicon cloned into *Hin*dIII/*Eco*RI digested binary vector pKEN (McGrath et al., 2005) and confirmed by sequencing. *35S::LBD20* pKEN was mobilized into *Agrobacterium tumefaciens* AGL1 and transformed into Arabidopsis Col-0 and *lbd20-2* as per McGrath et al. (2005). Transgenic plants were selected based on their resistance to 10 mg/L Pestanal (glufosinate ammonium; Riedel-de Haen) and resulting T2 and T3 lines were used in subsequent experiments.

F. oxysporum Inoculation

The *F. oxysporum* isolate used in this study was strain Fo5176 obtained from Dr. Roger Shivas, Queensland Plant Pathology Herbarium, Queensland Department of Primary Industries and Fisheries, Brisbane, Australia. Root-dip inoculations on 3- to 4-week-old plants with a 1×10^6 cell/mL spore suspension were performed as described (Campbell et al., 2003; Edgar et al., 2006; Thatcher et al., 2009).

qRT-PCR

qRT-PCR experiments were performed on tissue collected after mock, *F. oxysporum*, or MeJA treatment. For analysis of root and shoot tissues, plants were cut at the top of the root, just below the crown, so shoot tissue consisted of the hypocotyl and aerial tissue. Three biological replicates were taken in all experiments consisting of tissue samples pooled separately from 10 to 30 plants grown and treated at the same time in the same environment. For gene expression under MeJA treatment, 14-d-old plants were germinated on Murashige and Skoog plates then gently lifted into a mock medium (Murashige and Skoog broth) or 100 μM MeJA medium (Murashige and Skoog medium plus MeJA) such that the roots were submerged, and left for 6 h before harvesting. For experiments using *coi1*, homozygous *coi1* plants that are JA insensitive were selected on Murashige and Skoog agar plates containing 50 μM MeJA and at 7 d of age transferred to Murashige and Skoog-only medium. For all experiments, plants were gently lifted from the soil or broth, rinsed in water, blotted on filter paper, frozen in liquid nitrogen, and stored at −80°C. RNA extraction, complementary DNA synthesis, and qRT-PCR were conducted as described by McGrath et al. (2005) using an Applied Biosystems 7900HT fast real-time PCR system. Absolute gene expression levels relative to the previously validated (Anderson et al., 2004; Kidd et al., 2009; Thatcher et al., 2009) reference gene mix *β-actin2*, *β-actin7*, and *β-actin8* (At1g49240, At3g18780, and At5g09810, respectively) were used for each complementary DNA sample using the equation: relative ratio gene of interest/actin = (Egene$^{-Ct\ gene}$)/(Eactin$^{-Ct\ actin}$) where Ct is the cycle threshold value. The *β-actin* mix contains reverse primers for each of the three *β-actin* genes and a universal forward primer. The mean expression range of the reference gene was found to be within ±1 Ct across all samples. The gene-specific primer sequences have mostly been previously published (Anderson et al., 2004; McGrath et al., 2005; Edgar et al., 2006; Kidd et al., 2009; Thatcher et al., 2009) and are also listed in Supplemental Table S1.

F. oxysporum Culture Filtrate Assay

F. oxysporum culture filtrate assays were performed as per Thatcher et al. (2009) on 15 leaves per line. Lesion size was measured at 3 d post inoculation using the ImageJ freeware package (http://rsb.info.nih.gov/ij/).

Sequence data from this article can be found in the The Arabidopsis Information Resource data libraries under accession numbers At3g03760 (*LBD20*), At1g32640 (*MYC2*), At1g72260 (*Thi2.1*), At3g04720 (*PR4*), At5g24770 (*VSP2*), and At5g44420 (*PDF1.2*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *F. oxysporum* disease score ratings system.

Supplemental Figure S2. Analysis of *lbd20* T-DNA mutants.

Supplemental Figure S3. *PDF1.2* and *PR4* expression in wild-type versus *lbd20* plants following MeJA treatment.

Supplemental Figure S4. LBD20 is a repressor of a subset of JA-regulated defense genes following *F. oxysporum* infection.

Supplemental Figure S5. Plants overexpressing *LBD20* have altered leaf morphology and fertility.

Supplemental Table S1. qRT-PCR primers used in gene expression analyses.

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