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A MYB Transcription Factor Regulates Very-Long-Chain Fatty Acid Biosynthesis for Activation of the Hypersensitive Cell Death Response in *Arabidopsis* [W][OA]

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Plant immune responses to pathogen attack include the hypersensitive response (HR), a form of programmed cell death occurring at invasion sites. We previously reported on *Arabidopsis thaliana* MYB30, a transcription factor that acts as a positive regulator of a cell death pathway conditioning the HR. Here, we show by microarray analyses of *Arabidopsis* plants misexpressing *MYB30* that the genes encoding the four enzymes forming the acyl-coA elongase complex are putative MYB30 targets. The acyl-coA elongase complex synthesizes very-long-chain fatty acids (VLCFAs), and the accumulation of extracellular VLCFA-derived metabolites (leaf epidermal wax components) was affected in *MYB30* knockout mutant and overexpressing lines. In the same lines, a lipid extraction procedure allowing high recovery of sphingolipids revealed changes in VLCFA contents that were amplified in response to inoculation. Finally, the exacerbated HR phenotype of *MYB30*-overexpressing lines was altered by the loss of function of the acyl-ACP thioesterase FATB, which causes severe defects in the supply of fatty acids for VLCFA biosynthesis. Based on these findings, we propose a model in which MYB30 modulates HR via VLCFAs by themselves, or VLCFA derivatives, as cell death messengers in plants.

INTRODUCTION

Like many other complex biological processes, plant defense responses to pathogen attack involve transcriptional activation or repression of a large number of plant host genes. Many of these genes have been identified, and some have been shown to be essential for pathogen resistance. Those whose functions are known are involved in various defense mechanisms, including the generation of signals such as reactive oxygen intermediates, the synthesis of antimicrobial compounds, the lignification of cell walls and the production of cell wall degrading enzymes. Besides these general defense mechanisms, one of the most spectacular manifestations of plant resistance is the hypersensitive response (HR), a form of programmed cell death occurring in a limited area at the site of infection. Although the expression of pathogenesis-related (PR) genes and other defense genes has been shown to be associated with the HR, the molecular actors responsible for the control and execution of the HR cell death program remain mainly unknown. As in other stress responses (Singh et al., 2002; Shinozaki et al., 2003), the transcriptional regulation of plant genes plays a critical role in the control of this process. In fact, plant immune receptors can directly target some components of the transcriptional machinery activated for defense (Shen et al., 2007). In this context, we previously identified *MYB30*, an R2R3 MYB-related gene in *Arabidopsis thaliana* and a putative transcriptional factor that has been shown to act as a positive regulator of the programmed cell death associated with HR in *Arabidopsis* (Daniel et al., 1999; Vailleau et al., 2002).

MYB proteins are common to all eukaryotes, but in higher plants this protein family is extraordinarily large (Martin and Paz-Ares, 1997; Stracke et al., 2001). While MYB proteins from animals contain three repeats (R1, R2, and R3) of the MYB conserved domain, most plant MYB proteins are of the R2R3 type and are thought to be predominantly involved in plant-specific regulatory processes (Jin and Martin, 1999; Stracke et al., 2001). They have been implicated in a range of functions,

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including the phenylpropanoid pathway (Borevitz et al., 2000), morphogenesis (Glover et al., 1998), and abiotic (Abe et al., 1997) and biotic stress responses (Yang and Klessig, 1996; Geri et al., 1999; Sugimoto et al., 2000; Mengiste et al., 2003). Nevertheless, the functions of most of these R2R3 MYB proteins remain unknown. *MYB30* was identified in *Arabidopsis* on the basis of its specific, rapid, and transient transcriptional activation during the very first steps of the HR in response to the avirulent strain 147 of *Xanthomonas campestris* pv *campestris* (*Xcc*) and other bacterial pathogens (Daniel et al., 1999). The use of *Arabidopsis lsd* mutants (Daniel et al., 1999) and their corresponding suppressor *phx* mutants showed that *MYB30* expression is closely associated with the initiation of the HR rather than with its propagation. Furthermore, the analysis of transgenic plants showed that overexpression of *MYB30* accelerates the appearance of the HR in response to avirulent bacterial pathogens and causes HR-like responses to a virulent bacterial pathogen (Vailleau et al., 2002). In addition, it increases resistance against different bacterial and fungal pathogens, and antisense *MYB30 Arabidopsis* lines showed a reverse phenotype. An important role for salicylic acid (SA) has also been demonstrated for the control of the HR by MYB30 (Raffaele et al., 2006). MYB30 may thus be a component of a cell death pathway conditioning the HR, but its mode of action remains unclear.

In this study, we identified putative target genes for MYB30 using Affymetrix ATH1 microarrays and by comparing the gene expression profiles of *Xcc*-inoculated *Arabidopsis* plants from either overexpressor (*MYB30ox*) or antisense (*MYB30as*) transgenic lines with those of the wild type. Expression of these putative target genes was further analyzed by quantitative RT-PCR to confirm their expression patterns not only in the transgenic lines but also in a T-DNA insertion knockout (*MYB30ko*) mutant. Identified genes were found to be involved in very-long-chain fatty acid (VLCFA) biosynthesis pathways, suggesting the possible implication of this metabolism in the promotion of the HR. MYB30 was shown to directly activate the promoter of these genes by transient assays in *Nicotiana benthamiana*. In addition, the levels of very long acyl chains from a sphingolipid-enriched fraction were affected in the transgenic and mutant lines, and these alterations were exacerbated in response to pathogen challenge. Finally, loss of function of the thioesterase FATB reverses the accelerated and intensified HR phenotype of the *MYB30ko* line. Based on these findings, we propose a model in which MYB30 modulates cell death lipid signaling by enhancing the synthesis of VLCFA in the endoplasmic reticulum.

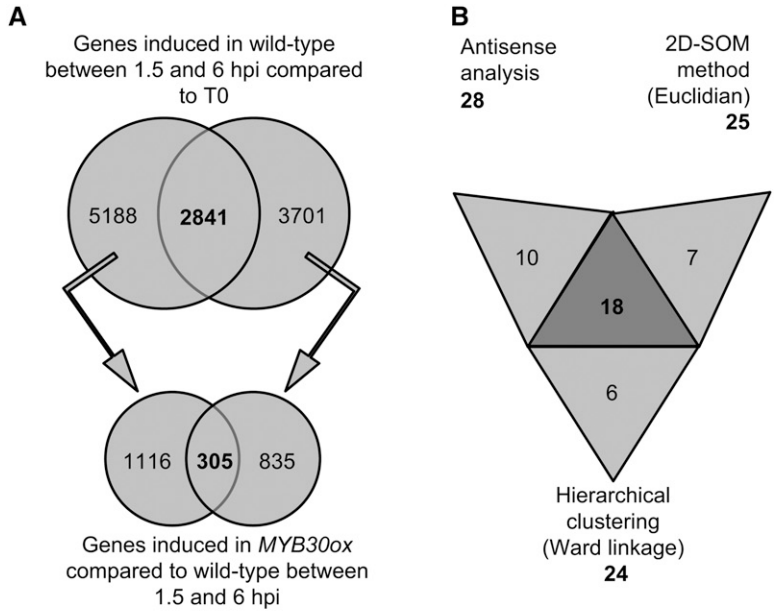
RESULTS

Strategy to Identify Genes That Act Downstream of At MYB30 by Whole-Genome Microarray Analysis

Oligonucleotide microarrays containing more than 24,000 genes (Affymetrix ATH1 GeneChip) were used to quantitatively assess changes in gene expression in the first hour following inoculation with the avirulent strain *Xcc*147. Two independent experiments were performed in which leaf samples of *Arabidopsis* wild-type and transgenic plants were harvested for RNA isolation 90 to 105 min, 2 to 4 h, and 6 h after inoculation. The *MYB30ko* line was not available at the time that these experiments were performed. Using MAS5.0 (MicroArray Suite; Affymetrix) to screen for candidate target genes (see Methods for details), we identified a set of 305 candidate genes (see Supplemental Table 1 online): (1) upregulated in the wild-type line after inoculation with *Xcc147* compared with T_0 time point and (2) overexpressed in the *MYB30ox* line compared with the wild type (Figure 1A). We then conducted three independent approaches to identify candidate coexpressed genes (Figure 1B). First, microarray data were used to select genes downregulated in the early time points (90 to 105 min after inoculation) in the *MYB30as* line compared with the wild type. A set of 28 genes was identified in two independent biological experiments. A preliminary functional classification of these 28 genes revealed that 50% are involved in lipid metabolism, while several other putative target genes are implicated in signal transduction or response to stress (see Supplemental Table 2 online). Second, unsupervised hierarchical clustering with Ward linkage and the Spearman rank correlation-based distance was performed on the same set of 305 genes. This statistical analysis by progressive regrouping allowed us to highlight a set of 24 genes, including 18 of the genes obtained with the previous approach (Figure 1B; see Supplemental Table 3 online), reinforcing the idea of a subset of coexpressed genes regulated by MYB30. Third, we clustered the 305 genes in a two-dimensional self-organizing map (2D-SOM) analysis using the GeneSight program (Biodiscovery). To focus on the shape of expression patterns rather than the magnitude of individual expression changes, the analysis was performed on the signal log ratio, corresponding to the ratio of the signal for each time point after inoculation compared with the T_0 time point. *MYB30* was added to the 305 candidate gene list to select genes presenting the same expression profile. Interestingly, this partitioning analysis identified a cluster containing *MYB30* and 19 out of the 28 genes obtained with the antisense approach (see Supplemental Table 4 online). In summary, we identified a set of 18 genes selected through the three approaches (Figures 1B and 1C), 14 of which have a function related to lipid metabolism. This indicates that we can indeed identify weakly differentially expressed genes by this method, as previously shown (Journot-Catalino et al., 2006).

The Acyl-CoA Elongase Complex Responsible for VLCFA Biosynthesis Is the Main Putative Target of MYB30 Regulation

Fatty acids (FAs) are emerging as important molecules that participate in diverse biological processes, including the regulation of different defense/cell death signaling pathways (Weber, 2002; Kachroo et al., 2003), so we decided to focus on the genes associated with FA metabolism. Among these 14 lipid-related genes (Figure 1C), the exact function of *LTP*, *LTP2*, and *PPT1* remains unclear, but all other candidates are related to cuticle biosynthesis. *CER2* and *WAX2*/*CER3* are involved in wax biosynthesis, whereas *GPAT4* (Li et al., 2007) and *ATT1* act in the metabolism of cutin. At least seven genes encode subunits of the acyl-CoA elongase complex responsible for the biosynthesis of VLCFAs. Elongation of FAs occurs in the endoplasmic reticulum and involves four successive reactions catalyzed by four



C

Probe set[a]	Identifier[b]		WT_1 / WT_0	Ox / WT
Lipid Metabolism				
261570_at	AT1G01120	KCS1 (β-ketoacyl-CoA synthase 1)	2,2	2,8
259430_at	AT1G01610	GPAT4 (Glycerol-3P acyl transferase)	2,2	1,7
261420_at	AT1G07720	KCS2 (β-ketoacyl-CoA synthase 2)	1,5	2,1
259592_at	AT1G27950	LTP (Lipid transfer protein, GPI anchored)	2,3	1,6
245199_at	AT1G67730	GL8 (β-keto acyl reductase - GLOSSY8)	1,5	1,7
267377_at	AT2G26250	FDH (β-ketoacyl-CoA synthase - FIDDLEHEAD)	1,6	1,6
266415_at	AT2G38530	LTP2 (nonspecific lipid transfer protein 2)	2,3	1,5
251796_at	AT3G55360	CER10 (enoyl-CoA reductase)	3,3	2,9
255690_at	AT4G00360	ATT1 (cytochrome P450 CYP86A2)	2,4	1,5
245612_at	AT4G14440	HCD1 (enoyl-CoA hydratase/isomerase)	2,6	2,3
254122_at	AT4G24510	CER2 (coA-dependent acyl transferase)	3,7	3,9
248812_at	AT5G47330	PPT1 (palmitoyl thioesterase)	9,0	2,5
250428_at	AT5G10480	PAS2 (tyrosine phosphatase-like protein)	2,4	1,8
247884_at	AT5G57800	WAX2/CER3 (fatty acids reductase putative)	2,2	2,1
Other functions				
254041_at	AT4G25830	integral membrane protein	1,9	2,2
252943_at	AT4G39330	mannitol dehydrogenase	1,9	2,8
Unclassified				
245783_s_a	AT1G35180	expressed protein	2,3	2,9
248028_at	AT5G55620	expressed protein	1,4	2,1

Figure 1. Changes in Gene Expression in At *MYB30* Plants after Inoculation with the *Xcc*147 Strain.

(A) Venn diagrams showing the distribution of overlapping and nonoverlapping early (1.5 to 6 h after *Xcc*147 inoculation [hpi]) induced candidate genes in inoculated wild-type plants compared with the T_0 time point and in inoculated *MYB30ox* plants compared with inoculated wild type. Two independent experiments (represented by each circle) were performed.

(B) Diagram of the output of analyses performed to identify candidate genes from among the 305 identified in **(A)**. The numbers under the heading refer to the total number of genes identified by that method of analysis. The number of genes identified by all three methods is shown in the middle of the diagram. Genes identified by two but not three of the analyses are not presented. Lists of genes included in this diagram are provided in Supplemental Tables 2 to 4 online. Antisense analysis: genes (from among the 305 candidates) that were downregulated in *MYB30as* plants. Hierarchical clustering: unsupervised hierarchical clustering with Ward linkage and the Spearman rank correlation-based distance (2D-SOM method).

(C) Consensus list of 18 genes cited above. [a]Affymetrix Probe set number. [b]Arabidopsis Genome Initiative number and corresponding putative function. The final column shows the fold changes in the mean expression levels from two independent experiments in inoculated wild type compared with the wild type at T_0 (WT_1/WT_0) and in the *MYB30ox* line compared with the wild type after *Xcc*147 inoculations (Ox/WT).

different proteins. The first step is the condensation of an acyl-CoA and a malonyl-CoA. This rate-limiting step is catalyzed by a condensing enzyme (β-ketoacyl-CoA synthase [KCS]), and 21 genes encoding such proteins have been annotated in the genome of *Arabidopsis*. The next three steps of the elongating process are catalyzed by a β-ketoacyl-CoA reductase, a β-hydroxy acyl-CoA dehydratase, and a trans-2,3-enoyl-CoA reductase, respectively. Each of these three reactions is related to one or two loci in the *Arabidopsis* genome. In Figure 1C, *KCS1*, *KCS2*, and *FDH* encode β-keto acyl-CoA synthases (Pruitt et al., 2000), *GL8* a β-ketoacyl-CoA reductase (Xu et al., 2002), *HCD1* and putatively *PAS2* hydroxy-CoA dehydratases (Denic and Weissman, 2007; Garcia et al., 2007), and *CER10* the trans-2,3-enoyl-CoA reductase (Zheng et al., 2005). These data suggest that genes responsible for VLCFA biosynthesis might be the first targets of MYB30.

To validate the response of these 14 genes to MYB30 deregulation and to assess the robustness of this response, we compared their expression levels by quantitative RT-PCR in five independent experiments using *MYB30ox*, *MYB30ko* (Raffaele et al., 2006), and wild-type lines in two different genetic backgrounds (Wassilewskija-4 [Ws-4] and Columbia-0 [Col-0]) and challenged by two different avirulent bacteria (*Xcc*147 and *Pst* DC3000/*avrRpm1*). In all the experiments performed, all 14 lipid-related genes were significantly induced at early time points of the interaction in the wild type, overexpressed in *MYB30ox*, and downregulated in *MYB30ko*, confirming the microarray-based expression data (Figure 2A; see Supplemental Figure 1 online). The transient and early pattern of induction of these genes fits well with a role in the onset of the HR and as putative targets of MYB30.

We also used quantitative RT-PCR to investigate whether upstream steps of the VLCFA pathway are affected in *MYB30* transgenic and mutant lines, looking at the expression patterns of a long-chain acyl-CoA synthetase gene (*LACS3*) and the *FATB* (acyl-ACP thioesterase B) gene after inoculation (Figure 2B). The expression patterns of these two genes are similar to those of the previous candidate target genes, suggesting that MYB30 regulation also affects the early steps of FA elongation.

At MYB30 Regulates the Expression of FA Elongase Complex Gene Promoters

To test directly the ability of At MYB30 to activate expression of these putative target genes, we used reporter constructs containing 1.3 to 2 kb of upstream sequences from different putative target genes fused to β-glucuronidase (GUS) and green fluorescent protein (GFP) in transient assays in *N. benthamiana* leaves. A construct containing At MYB30 without its activation domain (MYB30ΔAD) was used as a negative control. MYB30 led to an activation of the *pKCS1:GFP:GUS* and *pFDH:GFP:GUS* reporter constructs, showing up to a 5- to 10-fold increase in GUS activity compared with the reporter alone or to the reporter and MYB30ΔAD (Figure 3A). The other reporter gene (*GFP*) was also activated, as shown by fluorescence analysis (Figure 3B). By contrast, another lipid-related gene not identified as a putative target, *DH3* (At4g16210), was not activated under the same conditions (Figure 3A). Similar results were obtained with different putative target gene promoters (see Supplemental Figure 2 online). Immunodetection of TAP-tagged MYB30 and MYB30ΔAD showed that both proteins were expressed during the transient assays (Figure 3C). These results demonstrate that MYB30 is a potent activator of several genes involved in the early steps of VLCFA biosynthesis pathway.

At *MYB30* Deregulation Leads to Subtle Alteration of Epicuticular Waxes and Major Changes in the Intracellular VLCFA Content

Since MYB30 seems to control VLCFA synthesis, changes in leaf epicuticular waxes might occur in transgenic and mutant lines. Although *MYB30ox* leaves do not show a glossy phenotype, organ fusion, or any alteration in floral structure and fertility, we used (1) scanning electron microscopy to observe leaf surfaces, (2) measurement of surface permeability, and (3) gas chromatography–mass spectrometry (GC-MS) analysis of leaf and stem surfaces to assess any qualitative or quantitative changes in wax accumulation. No visible differences in cuticle thickness or pavement cell organization between *MYB30ox*, *MYB30ko*, and wild-type leaves were observed by scanning electron microscopy (see Supplemental Figures 3A and 3B online). Measurement of chlorophyll loss in ethanol showed no significant differences between transgenic *MYB30ox* leaves and wild-type leaves compared with *fatb-ko* mutant leaves (Figure 4A). Finally, GC-MS analysis performed on leaf material revealed a twofold increase of the most abundant leaf wax species, namely C29 and C31 alkanes, in MYB30-overexpressing lines compared with the wild type (Figure 4B). These alterations are consistent with the expression profiles of two wax-related genes (*CER2* and *WAX2*/*CER3*) that are more highly expressed in *MYB30ox* according to the microarray data and to quantitative RT-PCR analysis (Figure 4C). Since CER2 is involved in the elongation of FAs beyond C28 carbon atoms and WAX2/CER3 is involved in wax biosynthesis, wax and transcriptomic analyses are in agreement. Nevertheless, wax profiles show no difference between wild-type and *MYB30ko* lines (Figure 4B), and quantitative RT-PCR data indicate that expression of wax-related genes is not altered in *MYB30ko* (Figure 4C). These results suggest that other strong positive regulators of the wax biosynthesis pathway may hide *MYB30* misregulation of wax genes in *MYB30ko*. In addition, only weak differences between wax profiles of wild-type and *MYB30ox* were measured when extraction was performed on stems (see Supplemental Figure 3C online). Taken together, these data indicate that MYB30 may be a positive regulator of wax biosynthesis and accumulation but not a major one.

We next analyzed by GC-MS the FA composition (as a percentage of total FAs) in *MYB30* mutant and transgenic lines using a conventional chloroform/methanol extraction of lipids. A twofold increase was observed in the content in VLCFAs (sum of FAs from 20 to 26 carbon atoms) after inoculation. However, no significant differences could be detected in the different plant lines, healthy or after pathogen inoculation (see Supplemental Figure 3D online). Since sphingolipids are the major intracellular VLCFA-containing lipids in green parts of the plant and conventional lipid extraction methods underestimate the levels of

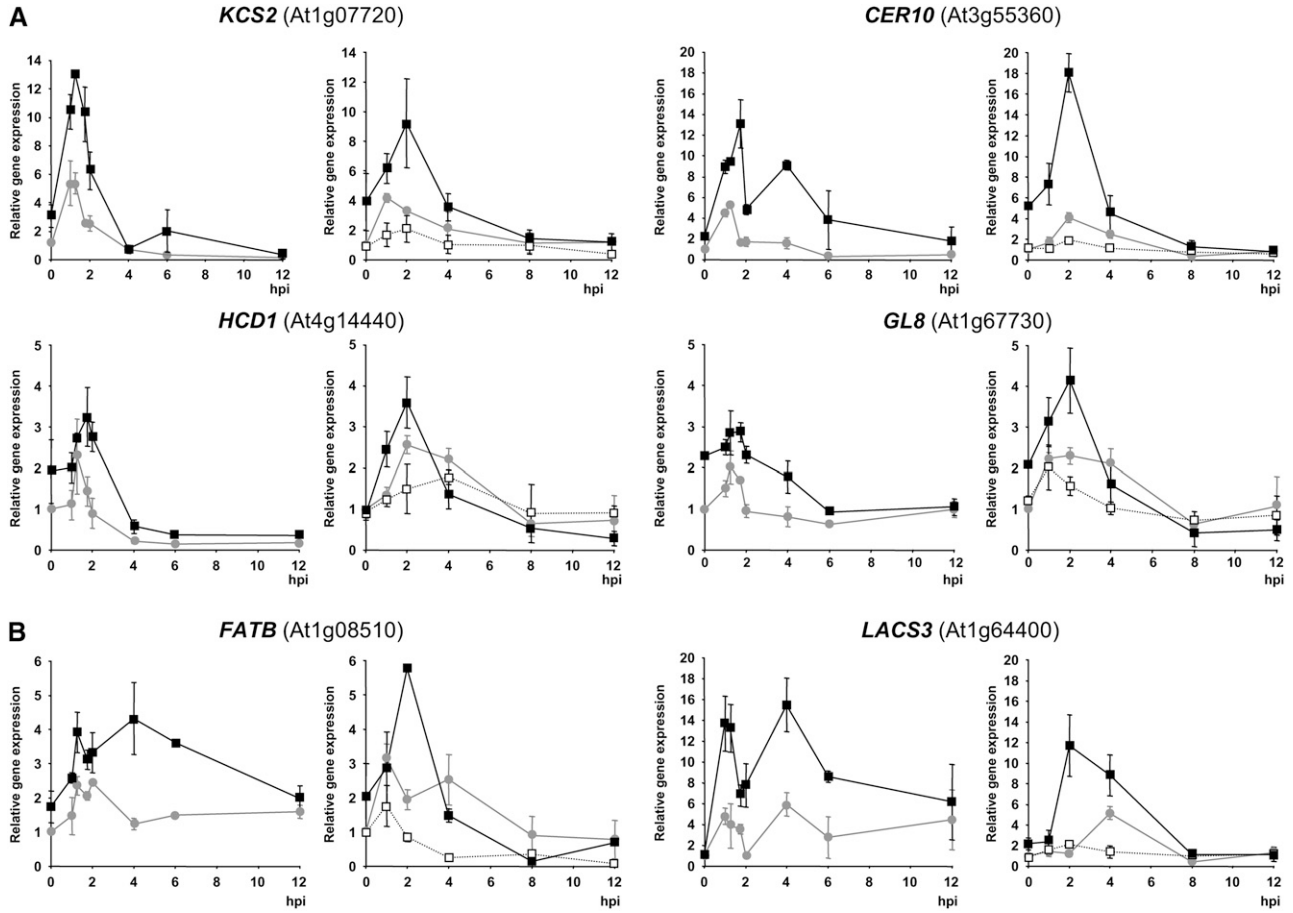


Figure 2. Effect of MYB30 Deregulation on the Expression of Pathogen-Induced, Lipid-Related Genes after Inoculation by Avirulent Bacterial Strains *Xcc147* (Left) and *Pst* DC3000/*avrRpm1* (Right).

(A) Expression pattern of four genes encoding each of the four subunits of the acyl-CoA elongase complex. These patterns are representative of those of the six genes involved in VLCFA synthesis tested (see Supplemental Figure 1 online).

(B) Expression pattern of two genes involved upstream of VLCFA biosynthesis.

Gene expression levels (determined by quantitative RT-PCR) are relative to the expression level in the wild type at T_0. Values represent mean ± SE from three independent experiments. Gray line with gray circles, the wild type; black line with black squares, *MYB30ox*; dotted line with open squares, *MYB30ko*. hpi, hours after inoculation.

sphingolipids, such as inositolphosphoceramide and glycosylinositol-phosphoceramides (Michaelson et al., 2002; Sperling et al., 2005), we used a method designed for extraction of the total sphingolipid content (Markham et al., 2006). As expected, VLCFAs are clearly identified by mass spectrometry (see Supplemental Figure 4 online), and yields are significantly higher than those obtained with the conventional method (Bligh and Dyer, 1959). Table 1 shows (1) an increase of the VLCFA content in wild-type plants after pathogen inoculation, (2) a slight increase of VLCFAs (+12%) in *MYB30ox* plants, which is enhanced after inoculation (+34%) compared with the wild type, and conversely, (3) a decrease of VLCFA content (−12%) in *MYB30ko*.

Thus, in noninoculated *MYB30ox*, MYB30 promotes the accumulation of VLCFAs, suggesting that MYB30 might be involved in potentialization of lipid signaling for defense/cell death. In response to inoculation, this regulator might promote VLCFA biosynthesis together with the use of VLCFAs and their derivatives for signaling.

The *fatB-ko* Mutation Reverses the Phenotypes of *MYB30ox*

The *Arabidopsis FATB* gene encodes an acyl-ACP thioesterase that participates in the determination of the amount and type of FAs exported from the plastids, and a mutation in this gene results in severe alteration in the supply of saturated FAs for VLCFA biosynthesis (Bonaventure et al., 2003). Because *MYB30ox* plants overexpress a number of genes involved in the early steps of VLCFA biosynthesis and contain higher levels of these FAs, we tested whether the phenotypes observed in *MYB30ox* could be suppressed by crossing *MYB30ox* with the *fatb-ko* mutant. A number of recessive *fatb-ko MYB30ox* double mutant lines were identified and tested by inoculation with

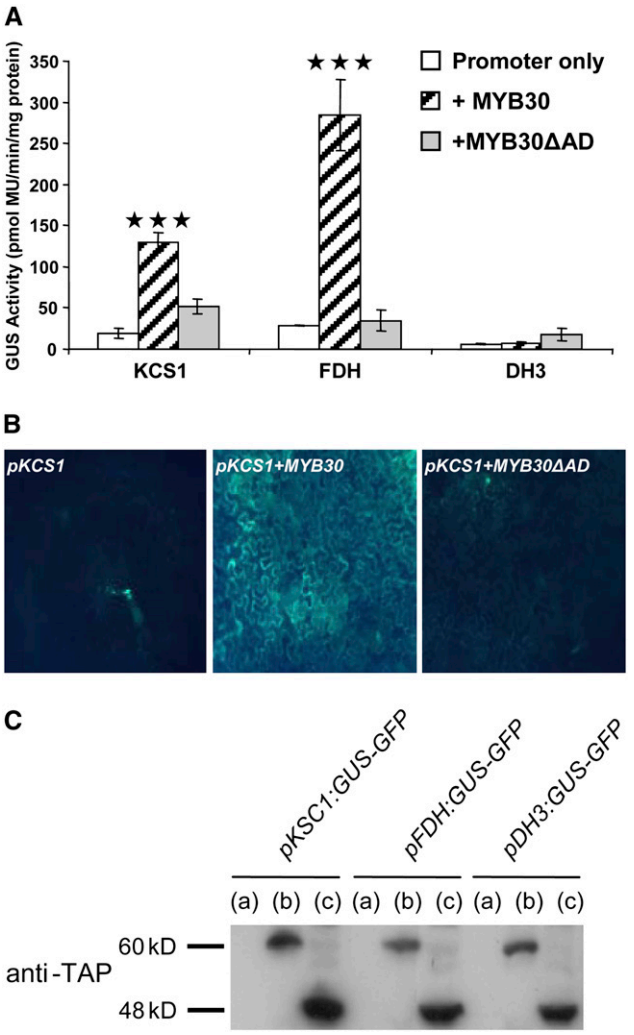


Figure 3. Transactivation of Lipid-Related Gene Promoters by At MYB30 in Transient Assays in *N. benthamiana* Leaves.

(A) GUS activity in leaf discs infiltrated with reporter constructs from the indicated gene either alone (white bars), with the MYB30 activator construct (hatched bars), or with the activator construct containing a truncated version of MYB30 (gray bars). These results are representative of at least six independent experiments. Mean ± SE from four replicates is shown. Statistical significance according to a Student's *t* test P value < 0.0005 is indicated by stars. MU, methylumbelliferone.

(B) GFP fluorescence of leaves 48 h after agroinfiltration with *pKCS1:GFP:GUS* alone or with an activator construct (as indicated).

(C) Immunodetection of TAP-tagged MYB30 and MYB30ΔAD 36 h after agroinfiltration. Proteins were extracted from infiltrated leaves and analyzed by protein gel blots using anti-TAP antibody. (a) Extracts from leaves after agroinfiltration with the indicated reporter construct alone; (b) extracts from leaves after agroinfiltration with the reporter and *MYB30* activator constructs; (c) extracts from leaves after agroinfiltration with the reporter and *MYB30ΔAD* activator constructs.

Pst carrying *avrRpm1* (Figure 5). Four days after inoculation, *MYB30ox* presented a clear HR, whereas the three double mutant lines tested and the parental line *fatb-ko* showed no necrotic symptoms (Figure 5A). Reversion of *MYB30ox*-conferred phenotypes by the presence of the *fatb-ko* mutant allele was confirmed by analysis of the expression of *PR1*, a defense marker gene upregulated in *MYB30ox* (Figure 5B). Double mutants resulting from crosses of *MYB30ox* with mutants affecting genes involved in other branches of FA biosynthesis pathways (*SSI2* and *FAD6*) were also tested by inoculation with *Pst* carrying *avrRpm1*. No detectable differences could be observed between *MYB30ox* and the different double mutant lines tested (Figure 5A), suggesting that reversion of *MYB30ox*-conferred phenotypes by the presence of the *fatb-ko* mutant allele is specific. This result demonstrates that the onset of MYB30-related phenotypes after inoculation is dependent on the supply of FAs for VLCFA biosynthesis. It supports the idea that (1) MYB30 target genes are involved in VLCFA biosynthesis and/or derivation and that (2) VLCFA and/or some of their derivatives are involved in establishment or control of the HR cell death program.

Echoes of MYB30 Regulation on the Biosynthesis of the Major Chloroplastic FAs and on the Oxylipin Pathway

To assess the specificity of the MYB30 effect on VLCFA biosynthesis-related genes, we tested whether deregulation of *MYB30* also alters the expression of genes regulating the synthesis of chloroplastic FAs. Seven genes, selected to cover a wide range of lipid metabolism reactions, were tested (Figure 6A; see Supplemental Figure 5 online). The three genes involved in de novo FA synthesis that we tested, namely *SSI2* (stearoyl desaturase), *MOD1* (enoyl-ACP reductase), and *ACC2* (acetyl-CoA carboxylase 2), showed no significant changes of expression in *MYB30ox* and *MYB30ko* compared with wild-type plants. However, two desaturase genes tested (*FAD8* and *FAD3*), responsible for biosynthesis of the major plastidial FA, were found to be repressed by MYB30 after inoculation. A significant induction of these genes is measured 4 h after inoculation in *MYB30ko* compared with wild-type plants. FA contents measured by GC-MS are in good agreement with these results. Both by classical chloroform/methanol (data not shown) and by sphingolipid-enriched extraction methods (Table 1) levels of unsaturated C16 FAs appear higher in *MYB30ko* (where *FAD* genes are induced) and lower in *MYB30ox* (where *FAD* genes are repressed) than in wild-type plants. This is particularly clear in *MYB30ox* pathogen-challenged plants where a lower level of unsaturated C18 FAs is measured. Based on these findings, we can assume that the chloroplastic pathways are affected as a consequence of a primary effect of MYB30 on the genes involved in VLCFA biosynthesis.

In the same vein, the ability of MYB30 to activate jasmonic acid (JA) production and JA-dependent defense responses was investigated. Modifications of oxylipin profiles in *MYB30* transgenic plants have been previously reported (Vailleau et al., 2002). Production of JA and of 12-oxo-phytodienoic acid (OPDA), a JA precursor, was evaluated by GC-MS (Figure 6B). JA and OPDA accumulated at similar levels in *MYB30ox* and the wild type during the first day after inoculation but showed a twofold

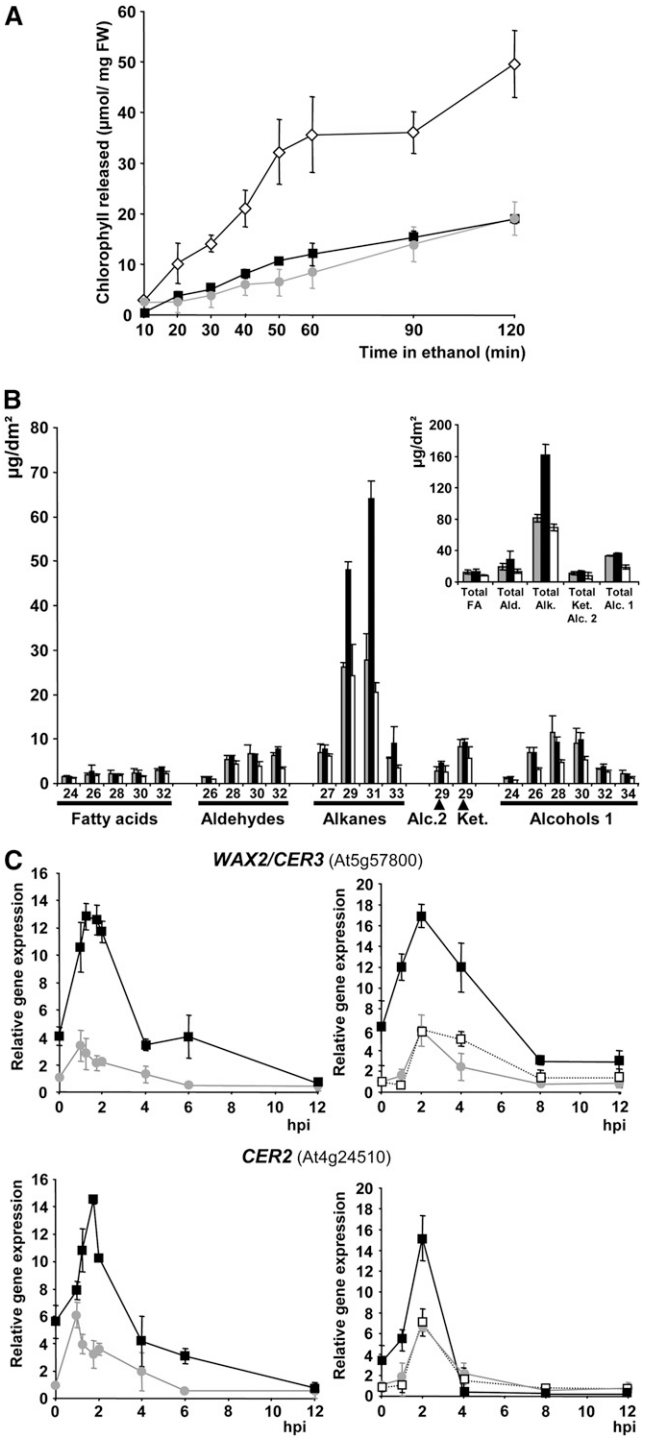


Figure 4. Alterations of *Arabidposis* Epicuticular Waxes by Modulation of MYB30 Expression.

(A) Chlorophyll leaching assays with mature rosette leaves immersed in 80% ethanol after different times. Gray line with gray circles, the wild type; black line with black squares, *MYB30ox*; black line with open diamonds, *fatb-ko* mutant. Values are mean ± SE from five replicates; one representative experiment out of two is shown.

(B) GC-MS quantification (expressed as µg/dm^2) of leaf epicuticular waxes from the wild type (gray bars), *MYB30ox* (black bars), and *MYB30ko* (white bars). The top right panel presents quantities of compounds grouped by molecular families. Ald., aldehyde; Alk., alkane; Ket., ketone; Alc. 2, secondary alcohol; Alc. 1, primary alcohol. Values represent means ± SE from five replicates; one representative experiment out of three is shown.

(C) Relative expression measured by Q-RT-PCR of two wax-related genes after inoculation by the avirulent strains *Xcc147* (left panels) and *Pst* DC3000/*avrRpm1* (right panels). Gene expression levels are quantified relative to the expression level in the wild type at time 0. Gray line with gray circles, the wild type; black line with black squares, *MYB30ox*; dotted line with open squares, *MYB30ko*. Values represent mean ± SE from three independent experiments.

decrease in *MYB30ox* 48 h after inoculation. However, production of both molecules was found to be reduced in the *MYB30ko* line after inoculation compared with the wild type.

In addition, pathogen-induced expression of two JA marker genes related to the pathogen response (*PR4* and *PDF1-2*) and two JA marker genes associated with the wound response (*VSP1* and *LOX3*) was monitored (Lorenzo et al., 2004) (Figure 6C). While the two first markers showed a clear decrease in their expression levels in *MYB30ox* compared with the wild type and were not altered or enhanced in *MYB30ko*, the second set of JA marker genes exhibited a reverse expression pattern in response to inoculation with *Pst* DC3000/*avrRpm1*. In addition, it should be noted that (1) the first alteration in oxylipin/JA signaling was observed only 4 to 8 h after inoculation, a few hours after expression of MYB30, (2) expression of genes encoding oxylipin synthesis enzymes, namely α-*DOX1* (dioxygenase) and *HPL1* (hydroperoxyde lyase), was not affected in the different *MYB30* lines compared with the wild type, and (3) several genes involved in jasmonate synthesis, such as *AOS* (allene oxide synthase), *OPR2* (oxo-phyto-dienoate reductase), and *JMT* (jasmonate methyl-transferase), showed no differential expression between plant lines (see Supplemental Figure 5 online). Finally, double mutants containing the *MYB30ox* construct and the *jar1* mutation that affects JA signal transduction (Staswick et al., 2002) showed the same accelerated and enhanced HR phenotype as *MYB30ox* plants after inoculation by an avirulent strain of *Pst* (Figure 6D). These results indicate that JA signal transduction does not play an essential role in MYB30-driven phenotypes.

All together, these results show that the genes involved in VLCFA biosynthesis may be direct downstream targets of MYB30, being specifically and very rapidly regulated by MYB30, and suggest a rather complex, and probably indirect, effect on the chloroplastic and JA pathways by MYB30.

DISCUSSION

At *MYB30* was identified as a gene rapidly, transiently, and specifically expressed during the HR (Daniel et al., 1999) and was shown by genetic approaches to be a key transcriptional activator of this response (Vailleau et al., 2002). One way of understanding the mode of action of this putative transcriptional regulator and, more generally, the molecular mechanisms involved in the regulation of the HR is to search for downstream

Table 1. Leaf FA Composition Measured after Solubilization by Method IV from Markham et al. (2006) in Wild-Type (Col) and *MYB30* Mutant *Arabidopsis* Plants.

Plant Line	Treatment	C16:0	C16:X	C18:0	C18:X	C>20
Wild type	None	21.57 ± 0.73	11.01 ± 0.46	1.94 ± 0.08	64.06 ± 0.37	1.43 ± 0.27
MYB30ko	None	23.14 ± 1.14	11.26 ± 0.29	2.00 ± 0.09	62.34 ± 0.54	1.24 ± 0.10
MYB30ox	None	23.20 ± 1.56	10.69 ± 0.60	2.04 ± 0.33	62.46 ± 1.36	1.60 ± 0.20
Wild-type	Versus *Pst* DC3000/*avrRpm1* (24 hpi)	26.63 ± 0.16	10.66 ± 0.08	1.70 ± 0.08	58.86 ± 0.21	2.14 ± 0.09
MYB30ko	None	26.02 ± 0.20*	11.90 ± 0.44*	1.64 ± 0.04	58.52 ± 0.22	1.90 ± 0.24*
MYB30ox	None	29.98 ± 0.24*	9.19 ± 0.50*	1.86 ± 0.04*	56.08 ± 0.75*	2.87 ± 0.24*

The contents in FA species are given as mean values with SD. C16:X and C18:X, unsaturated FAs with 16 and 18 carbon atoms, respectively; C>20, sum of FAs with 20 or more carbon atoms. Significant differences according to a Student's *t* test are marked by an asteriskwhen $P < 0.05$. Values shown are mol %. hpi, h after inoculation.

target genes of MYB30. Here, we show by microarray analysis of *Arabidopsis* plants that misexpress *MYB30* and exhibit an altered HR that the putative target genes of this regulator are related to a specific step of lipid biosynthesis pathways, elongation of FAs to form VLCFAs. These data link MYB30 to the acyl-CoA elongase complex, required to generate derivatives playing important regulatory roles in plants, such as sphingolipids.

MYB30-Regulated Genes Are Related Mainly to the VLCFA Biosynthesis Pathway

The transcription factor MYB30 was found to act as a positive regulator of the HR. We demonstrate here, using microarrays to conduct a broad survey for genes whose expression is affected by the activity of MYB30, that it is implicated in the regulation of FA biosynthesis. Our analysis allowed the identification of 14 coexpressed genes involved in FA biosynthesis/signaling that display differential expression in the various lines versus the wild type. For all of the genes tested, quantitative RT-PCR confirmed that their RNA levels were changed in response to modifications in *MYB30* expression. Only a few genes (28%) identified in our study as activated directly or indirectly by MYB30 have putative functions unrelated to FA biosynthesis (i.e., stress and signaling responses) or unknown functions. The very low percentage of transcription factors recovered is striking (set of 305 genes; see Supplemental Table 1 online) and suggests that MYB30 may act relatively directly in regulating downstream targets. In addition, our 14 candidates are very rapidly upregulated, between 1 and 4 h after inoculation. This is particularly interesting if we consider that direct downstream genes for MYB30 would have to satisfy the following criteria: (1) increased level of expression in the MYB30 sense transgenic line, and the reverse in the KO line, (2) significantly increased transcripts 2 to 4 h after inoculation since the expression of the MYB30 transcript reaches its maximum

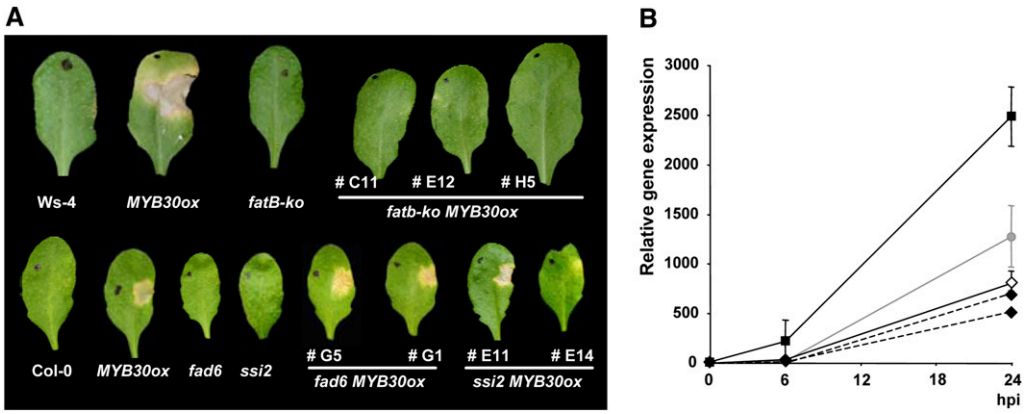


Figure 5. *fatb-ko* Reverses Phenotypes Conferred by *MYB30* Overexpression.

(A) Phenotype of *fatB-ko MYB30*ox, *fad6 MYB30ox*, and *ssi2 MYB30ox* double mutant lines 4 d after inoculation with *Pst* DC3000 carrying *avrRpm1* at low inoculum concentrations. Approximately 1 cm^2 surface of the right half of the leaves was syringe infiltrated. Selected double mutant lines showing representative symptoms are shown.

(B) Relative expression level of the *PR1* gene measured by quantitative RT-PCR after inoculation by *Pst* DC3000 carrying *avrRpm1*. Gene expression levels are quantified relative to the expression level in the wild type at time 0. Gray line with gray circles, the wild type; black line with black squares, *MYB30ox*; black line with open diamonds, *fatb-ko*; dotted line with black diamonds, *fatb-ko MYB30ox* double mutant lines. Values represent mean ± SE from three independent experiments.

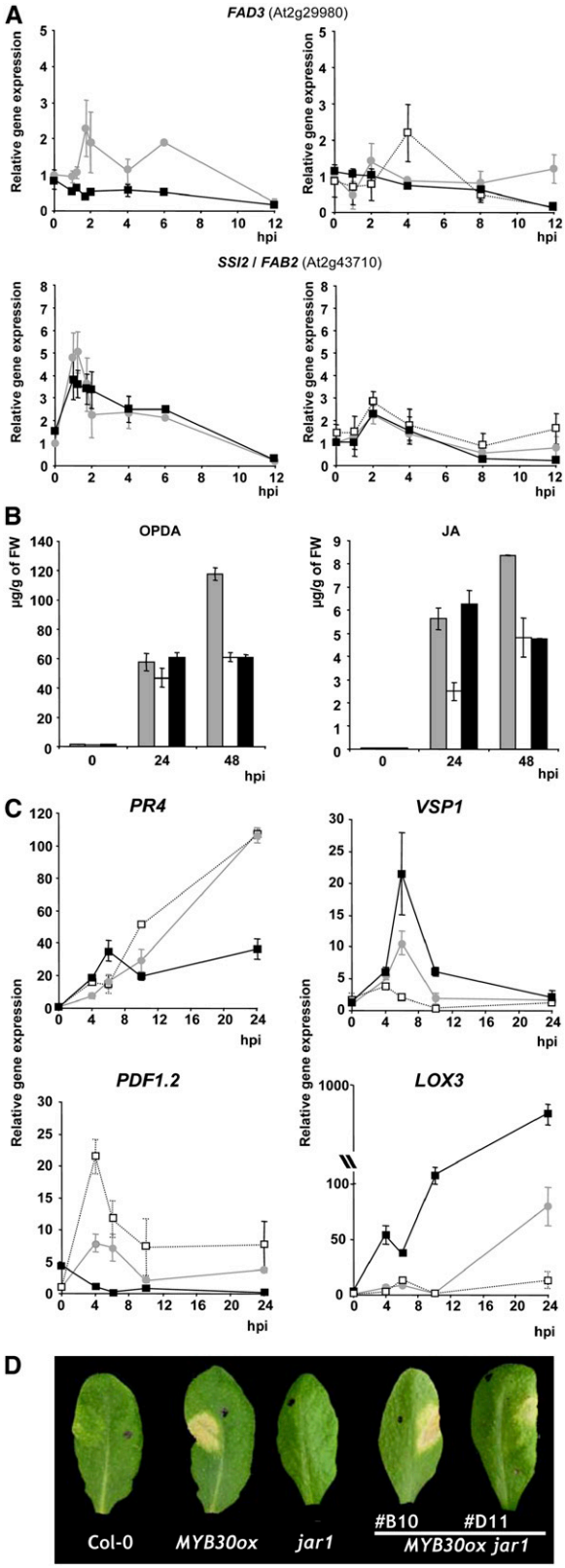


Figure 6. Relationships between MYB30 and FA Desaturation and the Jasmonate Signaling Pathways.

at 90 to 120 min after inoculation, and (3) existence of a MYB binding site motif in the promoter region. All of this evidence is compatible with MYB30 being a transcriptional activator of these genes during the HR. This is corroborated by transient expression assays where MYB30 behaves as a transcriptional activator of several genes encoding the four enzymes forming the FA elongase complex, responsible for VLCFA biosynthesis. This activity is dependent on the integrity of the C-terminal portion of MYB30, since its truncated version lacking the last 89 amino acids and putatively the activation domain was inactive in transient expression assays.

Contribution of Epicuticular Compounds to MYB30-Driven Phenotypes

WIN1 was reported as a transcriptional activator of epidermal wax accumulation in *Arabidopsis* (Broun et al., 2004). *LACS2* (long-chain acyl-CoA synthetase 2), a gene involved in cutin synthesis, is likely to be a direct target of WIN1 (Kannangara et al., 2007). WIN1 was shown to regulate a two-step process leading to changes in cutin composition: (1) a rapid and coordinated induction of cutin biosynthesis genes followed by (2) an induction of genes involved in wax biosynthesis. A parallel with MYB30 regulation can be established here, since MYB30 leads to (1) a rapid induction of genes involved in VLCFA synthesis, followed ~1 h later by (2) an induction of wax-related genes. However, while leaf epidermal wax accumulation was increased up to 4.5-fold in *WIN1*-overexpressing lines compared with the wild type (causing a glossy phenotype), MYB30 misexpression causes weaker, although consistent, phenotypes, as shown in

(A) Expression pattern of genes involved in plastidial FA biology: de novo PUFA (*FAD3*) and FA synthesis (*SSI2*) after inoculation by *Xcc147* (left panel) or *Pst* DC3000/*avrRpm1* (right panel). Gene expression levels (determined by quantitative RT-PCR) are relative to the expression level in the wild type at time 0. Gray line with gray circles, the wild type; black line with black squares, *MYB30ox*; dotted line with open squares, *MYB30ko*. Values represent mean ± SE from three independent experiments.

(B) GC-MS measurement of OPDA and JA in the wild type (gray bars), *MYB30ko* (white bars), and *MYB30ox* (black bars) plants after inoculation by the avirulent DC3000/*avrRpm1 Pst* strain. A representative experiment (out of two independent experiments) including means ± SD from three replicates is shown.

(C) Relative expression of JA pathway marker genes associated with wounding (*VSP1* and *LOX3*) and pathogen response (*PDF1-2* and *PR4*) by quantitative RT-PCR. Gene expression levels are quantified relative to the expression level in the wild type at time 0. Plants were inoculated by the avirulent DC3000/*avrRpm1 Pst* strain. Gray line with gray circles, the wild type; black line with black squares, *MYB30ox*; dotted line with open squares, *MYB30ko*. One representative experiment out of four is shown. Data for *MYB30ko* were standardized with the wild-type data to appear on the same graph.

(D) Phenotypes of *MYB30ox jar1* double mutants, parental lines, and the wild type 4 d after inoculation by *Pst* DC3000/*avrRpm1* at low inoculum concentrations. Approximately 1 cm^2 surface of the right half of the leaves was syringe infiltrated. Two double mutant lines out of five identified, showing representative symptoms, are shown.

Figure 4. It should be noted that no significant effect on wax synthesis could be detected in *MYB30ko*, neither at the gene expression level nor at the biochemical level, suggesting that the lack of *MYB30* activation can be overridden by stronger wax pathway activators in *MYB30ko* plants.

Nevertheless, we found among the list of 28 putative targets of MYB30 at least 13 genes related to cuticle synthesis. These were from different steps of cuticle assembly, from synthesis of precursor molecules to derivation and export from the cell. As previously discussed, the major functional group of genes encodes VLCFA synthesis enzymes, but we also identified two genes associated with cutin synthesis (*GPAT4* and *ATT1*) and two *LTP*s that could be involved in export of wax components out of the cell. This list of putative targets also contains two genes related to wax synthesis: *WAX2/CER3* and *CER2.* Interestingly, the role of the enzymes encoded by these two genes is not fully elucidated, and they are sometimes considered to be involved in FA elongation rather than wax assembly (Rowland et al., 2007).

By contrast, according to our microarray data, expression of the wax synthesis markers (*CER1* and *CER4*) is upregulated in healthy *MYB30ox* plants but falls down to the level of the wild type after inoculation (see Supplemental Figure 1B online). This is consistent with alteration of the wax content of healthy *MYB30ox* plants. These data support wax synthesis being altered as a consequence of targeting of the VLCFA synthesis in *MYB30* mutants. We hypothesize that in healthy plants overexpressing MYB30, VLCFAs, in higher abundance, are metabolized into wax component derivatives and that after inoculation, this pool of VLCFAs is redirected to the synthesis of derivatives involved in cell death signaling (Figure 7). Future work will be conducted to identify these putative signals.

Alterations of wax composition measured in *MYB30ox* could, however, account for a part of the phenotype of these plants. Indeed, in the *att1* mutant, cutin content was reduced to 30%, and disease severity following inoculation with *Pst* DC3000 was enhanced (Xiao et al., 2004). It was proposed that some FAs may

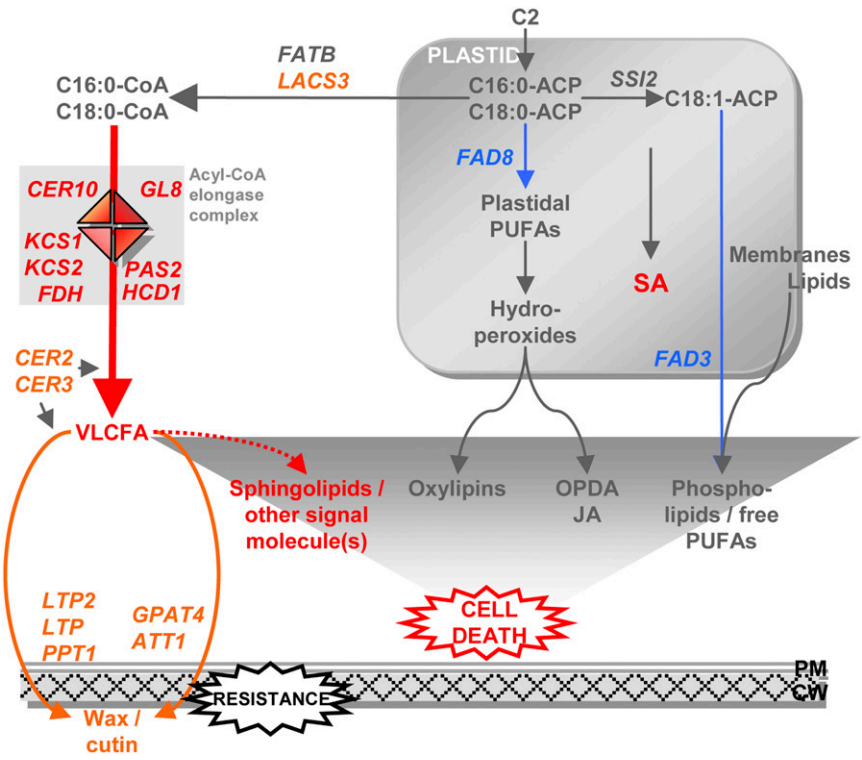


Figure 7. A Schematic Overview of Metabolic Pathways Regulated by MYB30 during the Incompatible Interaction between *Arabidopsis* and Avirulent Bacterial Pathogens.

Elements strongly (maybe directly) and positively regulated by MYB30 are indicated in red (genes of the acyl-CoA elongase complex, accumulation of VLCFAs, accumulation of SA, and cell death), other elements positively but weakly regulated by MYB30 are indicated in orange (upstream steps of VLCFA synthesis, wax- and cutin-related genes, and wax accumulation), elements shown in gray are not significantly regulated by MYB30, at least during the early events of the interaction, and elements shown in blue are negatively regulated (directly or indirectly) by MYB30 (expression of genes encoding FA desaturases). We hypothesize that inoculation triggers the synthesis of new signaling molecules as indicated by the dotted arrow. These molecules, together with other lipid-derived signals, such as salicylic acid, oxylipins, JA, and phospholipids, would subsequently act for activation of the hypersensitive cell death. Synthesis of these VLCFA-derivated signals may be enhanced by MYB30. Definitely, upregulation of VLCFA synthesis in nonchallenged *MYB30ox* plants allows an enhanced accumulation of VLCFAs and of VLCFA metabolizing enzymes. The cell is thus predisposed to respond stronger and faster to pathogen attack. PM, plama membrane; CW, cell wall.

repress bacterial type III gene expression in the intracellular space. Similarly, the *sma4* mutant, which is altered in *LACS2* gene encoding a member of long-chain acyl-CoA synthetases, displays severe symptoms when inoculated by *Pst* avirulent strains (Tang et al., 2007). However, this mutant shows high resistance to the necrotrophic fungal pathogen *Botrytis cinerea*, as do the *att1*, *bodyguard*, and *lacerate* mutants, all affected in cutin synthesis. Similar observations were made for the *bre1* mutant (Bessire et al., 2007). In these mutants, the more permeable cutin is assumed to allow a better release of antifungal compounds at the cell surface (Adie et al., 2007; Bessire et al., 2007). Thus, recent studies suggest that some extracellular lipid-derived molecules are able to repress the growth of pathogens, while others constitute a physical barrier to cell penetration. Although leaf epidermal wax accumulation is only weakly affected by *MYB30*, we hypothesize that activation of wax/cutin-related genes in *MYB30ox* may participate, to some extent, in the enhanced resistance phenotype (but not the HR phenotype) toward bacterial pathogens observed in these plants. In this context, our analysis suggested that resistance to the necrotrophic fungus *Sclerotinia sclerotiorum* is not (or only slightly) affected in the *MYB30* transgenic lines and in the *MYB30ko* mutant relative to the wild type (see Supplemental Figure 6 online). These data suggest that MYB30 has a complex function which can vary according to the interaction and that, as previously reported (Adie et al., 2007; Bessire et al., 2007), the complex regulatory network regulating plant defense is probably fine-tuned according to the particular characteristics of the interaction and not only to the pathogen lifestyles (biotrophic/necrotrophic).

An Integrated View of MYB30-Regulated Lipid Metabolism during the Incompatible Interaction

The FA composition of lipids has been shown to be affected after inoculation with avirulent bacteria (Yaeno et al., 2004), and defects in FA composition were shown to be responsible for altered defense responses in the *fad7 fad8* double mutant (Yaeno et al., 2004) and in the *ssi2* mutant, which fails to accumulate C18:1 (Kachroo et al., 2001). An explanation may be that polyunsaturated fatty acids (PUFAs) can modulate the activity of various regulatory enzymes (Ntambi and Miyazaki, 2003). We showed here that in the *MYB30* mutant and transgenic lines, the accumulation of PUFAs is altered along with the misregulation of *FAD3* and *FAD8*. Thus, the altered balance of saturated/unsaturated FAs by itself should be partly responsible for the phenotypes of the *MYB30* mutant and transgenic lines. On the other hand, oxilipins and jasmonates, generated from chloroplastic 18:3 and 16:3 FAs, were shown to play important regulatory roles in response to environmental signals (Turner et al., 2002; Weber, 2002; Farmer et al., 2003). MYB30 was shown to alter accumulation of these compounds: (1) tobacco plants expressing At *MYB30ox* exhibited markedly increased lipid peroxidation after inoculation with an HR-inducing pathogen compared with the wild-type plants (Vailleau et al., 2002), and (2) *Arabidopsis MYB30* mutant and transgenic plants show some weak and complex alterations of JA and OPDA accumulation after inoculation by an avirulent bacterial pathogen. However, despite a possible role of these molecules in the plant response to pathogens, the evidence accumulated in this work argues against direct regulation by MYB30 of the saturated/unsaturated balance of FAs and the oxylipin biosynthesis: (1) alteration (if any) of *FAD* and JA-related gene expression occurs a few hours later than the regulation of putative target genes, (2) accumulation of signaling compounds derived from chloroplastic FAs, such as oxylipins and jasmonates, do not show coherent and strong defects, (3) lipid peroxidation increase in *MYB30ox* (compared with the wild type) is coincident with lesion appearance (i.e., a few hours after putative target gene activation) (Vailleau et al., 2002), and (4) a mutation disturbing JA signal transduction does not alter MYB30-conferred phenotypes. These results suggest that modulation of some components of the chloroplastic FA biosynthesis pathway results more likely from a secondary effect of *MYB30* regulation on VLCFA synthesis.

To go further, different hypotheses can be proposed to integrate these secondary effects. First, considering that both VLCFAs and a part of plastidial PUFAs are synthesized from the same pool of C18:0, it can be conceived that alteration of VLCFA synthesis would impact plastidial PUFA metabolism. Besides, the *ssi2* mutant is affected in a plastidic stearoyl-ACP desaturase (Kachroo et al., 2001), which plays an important role in regulating the overall level of PUFAs and preferentially desaturates 18:0-ACP to yield 18:1-ACP. A reduction of C18:1 levels in *ssi2* plants results in constitutive activation of the SA-dependent pathway and in repression of the JA-dependent pathway (Kachroo et al., 2003), showing the importance of plastidial FAs in defense signaling and demonstrating the existence of an intricate signaling network involving FAs and both JA and SA pathways. Previous studies clearly established that SA- and JA-dependent pathways do not act independently and that key regulatory components may control crosstalk between these signaling pathways (Kachroo et al., 2003; Nandi et al., 2003; Li et al., 2004; Lorenzo et al., 2004). Consistent with this, MYB30 interferes with the ability of JA to activate *PDF1-2* and *PR4* expression but not *VSP1* and *LOX3* expression. These results suggest an antagonistic action of SA on some JA signaling pathways or a differential regulation of these two sets of genes, as previously shown (Lorenzo et al., 2004). Therefore, MYB30's primary effect (positive regulation of VLCFA synthesis) should also be considered as a part of an intricate signaling network conditioning defense responses.

To summarize, we show here that *MYB30* overexpression (1) coordinately enhances the expression of genes encoding the different subunits of the acyl-coA elongase complex required for VLCFA synthesis, (2) promotes the accumulation of VLCFAs inside the cell, (3) causes subtle changes in cuticle composition, (4) does not alter de novo FA biosynthesis, and (5) does not directly alter PUFA, JA, and oxylipin accumulation. Consequently, MYB30 may be involved in the generation of novel VLCFA-derived signals able to regulate the HR cell death and defense responses. A working model representing MYB30-dependent regulation of the different pathways related to FA metabolism in response to an avirulent pathogen is proposed (Figure 7).

MYB30: A Role in Lipid Signaling for Control of Cell Death?

Since regulation of the hypersensitive cell death response is one of the major phenotypes conferred by MYB30, and from the data

presented here, it is tempting to speculate that derivatives of the VLCFA biosynthesis pathway may participate in the regulation of programmed cell death.

Such molecules might be sphingolipids. Indeed, as discussed above, VLCFAs are required for synthesis of these ubiquitous membrane components in eukaryotic cells. Sphingolipids have a ceramide backbone, which consists of an FA attached to a long-chain amino alcohol. They have recently emerged as important signaling molecules regulating fundamental cell responses, such as cell death and differentiation, proliferation, and aspects of inflammation in animal cells. Ceramide generated in membranes has been shown to be central for the induction of apoptosis by death receptors and many stresses (Schenck et al., 2007). Ceramide-1-phosphate and ceramide are antagonistic molecules that can be interconverted in cells. Sphingosine-1-phosphate (S1P) is generated from ceramide by the consecutive actions of ceramidase and sphingosine kinase. S1P can induce cell proliferation via activation of the Edg family of receptors (for a review, see Gomez-Munoz, 2006). Plant ceramides are also able to induce cell death in *Arabidopsis* cells (Towney et al., 2005). The mutants *acd5* (Liang et al., 2003) and *acd11* (Brodersen et al., 2002), disrupted in a putative ceramide kinase and a sphingosine transfer protein, respectively, show increased disease symptoms during pathogen attack and apoptotic-like cell death, dependent on defense signaling. Interestingly, downregulation of the gene putatively encoding enoyl-CoA reductase that catalyzes the last step of VLCFA elongation in *N. benthamiana* produced typical cell death symptoms in leaves. Hence, regulation of the levels of these metabolites and their precursors, among them VLCFAs, is of the utmost importance in determining cell fate in animals and in plants.

In addition to signaling functions, VLCFAs could participate in membrane reorganization. In animal cells, ceramide forms large ceramide-enriched membrane domains that provide the spatial and temporal organization of the cellular signalosome upon activation. Membrane microdomains, also called lipid rafts, known to concentrate receptors, reorganize signaling molecules and thus amplify the primary signal generated by a receptor in animal cells, have been found to operate in response to pathogens (Stulnig et al., 2001; Gulbins et al., 2004). Similar structures have recently been found in tobacco leaves (Mongrand et al., 2004) and *Medicago truncatula* roots (Lefebvre et al., 2007). Therefore, MYB30 might modulate the HR cell death by promoting the generation of VLCFA- and/or plastidial FA-derived signals by altering the composition and organization of membrane lipid rafts (Mongrand et al., 2004).

In conclusion, this work identifies a novel role of the biosynthesis pathway of VLCFAs in controlling cell death and defense in plants. These findings set the stage for further investigation of molecular and biochemical mechanisms enabling plants to control cell death and select the right set of signaling pathways to respond to pathogen attack.

METHODS

Plant Materials

Arabidopsis thaliana plants were grown under controlled conditions as previously described (Lacomme and Roby, 1996). Plants from either the Ws genetic background (wild-type Ws-4, sense *pBIM131-20A-MYB30ox-*, and antisense *pBIW13-1A* transformed lines [Vailleau et al., 2002]) or the Col-0 genetic background (wild-type Col-0, sense *pBIM131-A1-MYB30ox-*, and the GABI-KAT T-DNA line 022F04-*MYB30ko*-) were used depending on the experiment, as detailed below.

Microarray Data and Statistical Analysis

Leaves of 4-week-old plants from the Ws-4 genetic background were syringe-infiltrated, with the *Xcc*147 strain, at 10^8 colony-forming units (cfu)/mL. For each time point, five leaves of 10 plants, noninoculated or inoculated, were harvested (60 min, 75 min, 90 min, 105 min, 2 h, 4 h, 6 h, 12 h, 24 h, and 30 h after infiltration), pooled (60 to 75 min, 90 to 105 min, and 2 to 4 h) and frozen. Total RNA was isolated as described (Lorrain et al., 2004). Labeling, hybridization, and detection on the ATH1 *Arabidopsis* chips (Affymetrix) were performed at the Affymetrix Platform from the Génopole Alsace-Lorraine (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France), as described (Redman et al., 2004). Genomic ratios were calculated with the Affymetrix Gene Chip software MAS 5.0 as described (Journot-Catalino et al., 2006). First, MAS 5.0 expression estimates were obtained in two independent experiments, and data were sorted with Excel and Access (Microsoft). Hierarchical clustering was done and the distance metric used was $d = (1 - \rho)$, with ρ being the Spearman rank correlation function between samples (Lucas and Jasson, 2006). The Ward linkage method was used, and the hierarchical tree was cut into 44 clusters. We also clustered our data into a 2D-SOM (Kohonen, 1990) 5×5 using the GeneSight program (Biodiscovery) with a Euclidian distance on signal log ratio. The data discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE9674.

RT-PCR

Leaves of 4-week-old plants were syringe-infiltrated by either the *Xcc*147 strain at 10^8 cfu/mL (experiments from Figures 2 and 4) or the *Pst* DC3000/*avrRpm1* strain at 5.10^7 cfu/mL (experiments from Figures 5 and 6). Total RNA extraction was performed from two leaf discs from two different plants for each experiment (Lorrain et al., 2004). Real-time quantitative PCR was performed as previously described (Lorrain et al., 2004) using SYBR green for DNA staining, and the primers and conditions used are described in Supplemental Table 5 online.

JA Extraction and Analysis

Leaves of 4-week-old plants from the Col-0 genetic background were syringe-infiltrated with the *Pst* DC3000/avrRpm1 strain at 5.10^7 cfu/mL. Tissue (500 mg) was used and the assay was performed as previously described (Stenzel et al., 2003) with the following modifications: HPLC fractions recovered were at retention times 13 to 14.5 (JA) and 21.75 to 22.5 (OPDA). Pentafluorobenzyl esters were measured with GC-MS on a Polariz Q (Thermo-Finnigan) at 100 eV with column 30 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness, cross-bond 5% diphenyl – 95% dimethyl polysiloxane, injection and interface temperatures 200 and 250°C, respectively, and oven program of 1 min at 60°C, 25°C min^{-1} to 180°C, 5°C min^{-1} to 270°C, 10°C min^{-1} to 300°C, and 10 min 300°C.

Transient Expression Assay in *Nicotiana benthamiana*

Transient expression assays were performed using *N. benthamiana* leaves as previously described (Rivas et al., 2002). Promoter regions of *KCS1*, *FDH*, and *DH3* genes (2010, 1963, and 1307 bp, respectively) were amplified from *Arabidopsis* genomic DNA using the following primers:

KCS1 promoter F and R for KCS1, FDH promoter F and R, DH3 promoter F and R for DH3 (see Supplemental Table 5 online). The corresponding PCR fragments were cloned into the pDONR207 ENTRY vector by Gateway recombinational cloning technology using the attB × attP (BP) recombination sites. Fragments containing promoter sequences were subsequently transferred into the pKGWFS7 Destination vector (Karimi et al., 2002) by LR cloning, resulting in plant expression vectors containing transcriptional fusions between the promoters and the *GUS* and *GFP* reporter genes. For construction of the activator plasmid, At *MYB30* and At *MYB30* without its transactivation domain (*MYB30ΔAD*, deletion from 234 to 323 amino acids) were amplified from *Arabidopsis* genomic DNA by PCR using the following primers: MYB30-ClaI-S and MYB30-BamHI-AS or MYB30ΔAD-BamHI-AS (see Supplemental Table 5 online), respectively, for *MYB30* and *MYB30ΔAD*. Following restriction enzyme digestion, they were ligated into the binary vector pBIN19H (Rivas et al., 2002). These constructs were introduced into GV3101 or C58C1 strains of *Agrobacterium tumefaciens*

GUS activity was assayed using the substrate 4-methylumbelliferyl-β-D-glucuronide as previously described (Balagué et al., 2003). GFP fluorescence was observed 2.5 d after agroinfiltration, using a fluorescence microscope (Leica) with a filter set providing 455 to 490 nm excitation and emission above 515 nm. Protein extraction was performed as previously described (Rivas et al., 2002) on four leaf discs per construct, harvested 1.5 d after inoculation. For electrophoresis, samples were loaded on NuPAGE Novex Bis-Tris gel as described by the manufacturer (Invitrogen). Proteins were separated and transferred onto nitrocellulose by wet electroblotting. For TAP protein detection, rabbit PAP soluble complex antibody (Sigma-Aldrich) was used as a diluted solution (at 1:2000) in TTBS (Tween-Tris buffered saline) + 5% skimmed milk powder. Blot was developed using the Immobilon kit (Millipore) by autoradiography.

Leaf Total FA Analysis

Leaves of 4-week-old plants from the Col-0 genetic background were syringe-infiltrated with the *Pst* DC3000/*avrRpm1 strain* at 5.10^7 cfu/mL. Each sample was harvested in five replicates, and analysis was performed on three independent biological experiments. For extraction of FAs, lipid solubilization was performed as in Method IV from Markham et al. (2006). From 1 g fresh weight of leaf tissue, 200 μL of the resulting lipid solution was evaporated under nitrogen and 40 μg of C17:0 was added as an external standard. Samples were then transmethylated in methanol–HCl for 18 h at 80°C. Cuticular waxes were extracted from fresh leaves by immersing tissues for 30 s in 15 mL of chloroform. After the addition of 5 μg of docosane as an internal standard, extracts were dried under a gentle stream of nitrogen, dissolved into 100 μL of BSTFA-TMCS [N,O-bis(trimethylsilyl)trifluoroacetamide):trimethylchlorosilane (99:1)] and derivatized at 80°C for 1 h. Surplus BSTFA-TMCS was evaporated under nitrogen and samples were dissolved in 200 μL hexane for analysis. An Agilent 6850 gas chromatograph with helium as the carrier gas (1.5 mL/min) was used. The gas chromatograph was programmed with an initial temperature of 160°C for 1 min and increased at 50°C/min to 200°C, held for 0.2 min at 200°C, increased again at 4°C/min to 320°C, and held for 8 min at 320°C. Qualitative analyses were performed using an HP-5MS column (30 m × 0.25 mm × 0.25 μm) and an Agilent 5975 mass spectrometric detector (70 eV, mass-to-charge ratio of 50 to 750). Quantitative analyses were performed using an HP-1 column (30 m × 0.32 mm × 0.25 μm) and a flame ionization detector. Quantification was based on peak areas and internal standards.

Genetic Analysis

Arabidopsis fatB-ko mutant (Bonaventure et al., 2003) and *MYB30ox* plants from the Ws-4 genetic background were crossed using *MYB30ox* as the pollen donor. F2 plants were screened by PCR to identify homozygous *fatB-ko* plants containing the *MYB30ox* construct. The same crossing procedure was used to generate *fad6 MYB30ox*, *ssi2 MYB30ox*, and *jar1 MYB30ox* double mutant plants with a Col-0 background. The *fad6* and *ssi2* mutants are SALK accessions, numbered N207 and N536854, respectively. Screening for homozygous mutations was performed using cleaved-amplified polymorphic sequence (Glazebrook et al., 1998) and the *Stu*I restriction site created by the *jar1* mutation, or the *Alw*NI restriction site, abolished by the *fad6* mutation. For phenotype observations, plants were infiltrated with a syringe on a limited area of the leaf with the *Pst* DC3000/*avrRpm1* strain at 5.10^5 cfu/mL.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: KCS1 (β-ketoacyl-CoA synthase 1) At1g01120, GPAT4 (glycerol-3P acyl transferase) At1g01610, KCS2 (β-ketoacyl-CoA synthase 2) At1g07720, LTP (lipid transfer protein, GPI anchored) At1g27950, GL8 (β-keto acyl reductase; GLOSSY8) At1g67730, FDH (β-ketoacyl-CoA synthase; FIDDLEHEAD) At2g26250, LTP2 (nonspecific lipid transfer protein 2) At2g38530, CER10 (enoyl-CoA reductase At3g55360, ATT1 (cytochrome P450; CYP86A2) At4g00360, HCD1 (enoyl-CoA hydratase/isomerase) At4g14440, CER2 (CoA-dependent acyl transferase) At4g24510, PPT1 (palmitoyl thioesterase) At5g47330, WAX2/CER3 (fatty acid reductase putative) At5g57800, FATB (acyl-ACP thioesterase B) At1g08510, LACS3 (long-chain acyl-CoA synthetase) At1g64400, DH3 (enoyl-CoA hydratase) At4g16210, FAD3 (fatty acid desaturase 3) At2g29980, SSI2/FAB2 (stearoyl desaturase 2) At2g43710, *MOD1* (enoyl-ACP reductase) At2g05990, *ACC2* (acetyl-CoA carboxylase 2) At1g36180, α-DOX (α-dioxygenase) At3g01420, AOS (allene oxide synthase) At5g42650, *OPR2* (oxo-phyto-dienoate reductase) At1g76690, *JMT* (jasmonate methyltransferase) At1g19640, HPL (allene oxide synthase) At4g15440, and FAD8 (fatty acyl desaturase 8) At5g05580.

Supplemental Data

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

Supplemental Figure 1. Effect of MYB30 Deregulation on the Expression of Genes Associated to VLCFA Metabolism after Bacterial Inoculation.

Supplemental Figure 2. Transactivation of Lipid-Related Gene Promoters by MYB30 in Transient Assays in *Nicotiana benthamiana* Leaves.

Supplemental Figure 3. Additional Information about Alterations of *Arabidopsis* Epicuticular Waxes by Modulation of MYB30 Expression.

Supplemental Figure 4. Identification by GC-MS of Lipid Acyl Chains in Wild-Type and *MYB30ox* Lines Inoculated by *Pst* DC3000/*avrRpm1*.

Supplemental Figure 5. Effect of MYB30 Deregulation on the Expression of Jasmonate- and Other Plastidial Lipid-Related Genes after Inoculation by Avirulent Bacterial Strains *Xcc*147 and *Pst* DC3000/*avrRpm1*.

Supplemental Figure 6. *Sclerotinia sclerotiorum* Infection of At MYB30 Lines.

Supplemental Table 1. List of the 305 Candidate Genes Differentially Expressed in Wild-Type and MYB30-Overexpressing Plants at Different Time Points after Inoculation with *Xcc*147.

Supplemental Table 2. The 28 Genes That, upon Inoculation with *Xcc*147, Are Induced in the Wild Type, Upregulated in *MYB30ox*

Compared with the Wild Type, and Repressed in *MYB30as* Compared with the Wild Type.

Supplemental Table 3. The 24 Genes Identified with Hierarchical Clustering with Ward Linkage and the Spearman Rank Correlation Based Distance (Lucas and Jasson, 2006).

Supplemental Table 4. The 25 Genes Identified with a 2D-SOM (Kohonen, 1990) 5 × 5 Using the GeneSight Program with a Euclidian Distance on Signal Log Ratio.

Supplemental Table 5. Primers Used in This Work.

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A MYB Transcription Factor Regulates Very-Long-Chain Fatty Acid Biosynthesis for Activation of the Hypersensitive Cell Death Response in *Arabidopsis*

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