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Title: GhERF-IIb3 regulates accumulation of jasmonate and leads to enhanced cotton resistance to blight disease

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Accepted Article

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ABSTRACT

The phytohormone jasmonic acid (JA) and its derivatives, collectively referred to as jasmonates, regulate many developmental processes, but are also involved in response to numerous abiotic/biotic stresses. Thus far, powerful reverse genetic strategies employing perception, signaling or biosynthesis mutants have broadly contributed to our understanding of JA implication in plant stress response and development; so did the chemical gain-of-function approach based on exogenous application of the hormone. However, there is currently no method that allows for *in planta* tightly controlled JA production. Investigating the control of the JA synthesis pathway in bacteria-infected cotton (*Gossypium hirsutum* L.) plants, we identified a transcription factor (TF), namely *GhERF-IIb3*, that could act as positive regulator of the JA pathway. Expression of this well-conserved TF in cotton leaves was sufficient to bring about *in situ* JA accumulation at physiological concentrations associated with enhanced cotton defense response to bacterial infection.

INTRODUCTION

Phytopathogen attacks result in major agricultural concerns. Diseases caused by pathogens in the field and at the post-harvest stages are indeed responsible for considerable losses representing approximately one third of the world production of crop species. The annual cotton plant, providing fibers for numerous downstream industrial applications, is not safe from such a plague. Bacterial blight triggered by *Xanthomonas citri* pv. *malvacearum* (*Xcm*) is the most damaging disease for cotton plants, affecting leaves and subsequently reducing cotton fiber yield (Delannoy *et al.*, 2005). Although attention is currently paid to genetically improved resistance to *Xcm* (Essenberg *et al.*, 2014), molecular mechanisms underlying cotton resistance to *Xcm* remains poorly understood. In the cotton cultivar Réba B50 that contains the *R* genes *B₂B₃*, the avirulent *Xcm* strain (named race 18) induces effector-triggered immunity (ETI, Cui *et al.*, 2014) accompanied by a typical Hypersensitive Reaction (HR) phenotype. Several molecular traits associated with cotton HR were characterized. These include early production of reactive oxygen species (Martinez *et al.*, 1998), enzymatic lipid peroxidation (Jalloul *et al.*, 2002), accumulation of salicylic acid (SA) correlated with the onset of systemic acquired resistance (Martinez *et al.*, 2000), up-regulation of peroxidase-encoding genes (Delannoy *et al.*, 2003), synthesis of antimicrobial compounds like flavonoids (Dai *et al.*, 1996) as well as some phytoalexins belonging to the sesquiterpene family (Essenberg *et al.*, 1990). More recently, JA signaling has been also associated with cotton HR (Champion *et al.*, 2009).

Aside from its functions in plant growth and development, JA is known to be involved in diverse physiological processes like programmed cell death, response to mechanical injury and plant-pathogen interactions (Wasternack and Hause, 2013). In response to stress, neo-synthesis of JA is initiated in plastids by 13-lipoxygenases (LOX) that catalyze oxidation of free and/or membrane-esterified linolenic acid. Resulting fatty acid hydroperoxides are then

sequentially processed by allene oxide synthase (AOS) and allene oxide cyclase (AOC) to produce oxo-phytodienoic acid (OPDA). OPDA is in turn converted into JA by reduction and three rounds of β -oxidation; the latter steps of which invoke acyl-coA oxidases (ACX) likely localized to peroxisomes. This metabolic pathway, known as the octadecanoic pathway, can be completed in the cytoplasm where JA is conjugated to the amino acid isoleucine (Ile) to form JA-Ile, which is bioactive (Lee *et al.*, 2008; Fonseca *et al.*, 2009). Of note, OPDA can also be esterified on structural glycerolipids (namely arabidopsides) and may be directly mobilized to produce JA under stressful conditions (Kourtchenko *et al.*, 2007).

Transcriptional regulation of JA-responsive genes relies on a core protein complex, the main components of which are the F-box protein CORONATINE INSENSITIVE1 (COI1) protein, the JASMONATE ZIM DOMAIN (JAZ) transcriptional repressors and TFs (Pauwels and Goossens, 2011). Basically, in the absence of JA-Ile, target gene expression is constitutively repressed due to TF and JAZ protein interaction within the TOPLESS (TPL)-NOVEL INTERACTORS OF JAZ (NINJA) repressor complex, which prevents TFs from binding to promoters (Pauwels *et al.*, 2010). When JA-Ile is produced, JAZ proteins are recruited by COI1 through indirect interaction with the hormonal conjugate, ubiquitinated and rapidly degraded in a proteasome-dependent manner (Larrieu *et al.*, 2015). Freeing TFs from their constraint thus results in transcriptional activation of JA-responsive genes, thereby launching JA signaling cascade and downstream physiological responses.

JA perception and signaling is well documented. However, it is still not clear how JA synthesis is activated in plants. Only few regulatory components have been identified thus far (S eo et al. 1995, 1999; Ellis & Turner 2001; Ellis et al. 2002; Schommer et al. 2008; Ito et al. 2007). Here, we describe that a novel TF, namely GhERF-IIb3, which belong to the Ethylene-Response Factor (ERF) family, is rapidly and transiently up-regulated in response to the avirulent *Xcm* strain. Using protoplast assays, we have shown that this TF is localized to

nucleus and is able to trans-activate the Arabidopsis AOC2 promoter in a sequence-specific manner. Furthermore, we also demonstrate that this TF is sufficient to bring about jasmonic acid accumulation *in planta* to enhance defense response to bacterial pathogens like *Xcm*.

RESULTS

Identification of genes potentially involved in jasmonate biosynthesis in cotton plants

Before examining the expression of genes coding for octadecanoic enzymes in response to the bacteria *Xcm*, we first searched for the corresponding coding sequences in dedicated genomic databases (see material and method section). Highly similar orthologs of AtAOS, AtAOC2 and AtACX1a were identified using *A. thaliana* primary amino-acid sequences as queries (Supplemental Table 1). To validate our candidate genes, their expression was quantified in wounded cotton cotyledons using a hemostat to promote JA accumulation *in planta*. Wounded tissues were then harvested over a time-period of 24h and the expression of *GhAOS-like*, *GhAOC2-like* and *GhACX1a-like* genes was quantified by qRT-PCR. As expected, wounding triggered a marked increase in JA content preceded by a transient accumulation of its precursor OPDA, as evidenced by LC-MS measurement (Figure 1A). A close correlation between the kinetic induction of the three candidate genes and that of the octadecanoic metabolites was also obtained in wounded tissues (Figure 1B).

To further reinforce these findings, their expression was quantified in JA-treated cotyledons because genuine JA biosynthesis genes are known to undergo a positive transcriptional regulatory loop (Browse, 2009). In accordance with their postulated function, all three identified cotton genes exhibited a significant induction at 2 h following application of 50 μ M JA compared to solvent treated-controls. Moreover, these genes, like others that respond to JA (Van der Does *et al.*, 2013), were not regulated by a 50 μ M SA treatment²⁶ (Supplemental Figure 1).

On the basis of these results, *GhAOS-like*, *GhAOC2-like* and *GhACX1a-like* gene expression, together with metabolite levels, were thus followed as hallmarks for potential activation of octadecanoic pathway in further analysis in cotton plants.

Jasmonate and its precursor, OPDA, specifically accumulate during cotton HR

GhAOS-like, *GhAOC2-like* and *GhACX1a-like* expression profiles were determined by qRT-PCR. The cotton cultivar Reba B50, carrying the *B₂B₃* blight-resistance genes, was used for that purpose. While this cultivar shows a resistance phenotype to *Xcm* race 18, characterized by a rapid and localized HR, it develops disease symptoms following inoculation with *Xcm* race 20 (Innes, 1983). During the incompatible interaction, steady-state levels of *GhAOS-like*, *GhAOC2-like* and *GhACX1a-like* transcripts increased significantly at 8 and 16 hpi (hours post-inoculation) when compared to those of untreated control and compatible tissues (Figure 2A). Moreover, this stimulation of gene expression appeared to be specific to HR-developing cotyledons.

To corroborate the transcriptional analysis, foliar concentrations of OPDA and JA upon *Xcm* challenge were then measured. Consistent with the expression kinetics of the three putative octadecanoic genes, both OPDA and JA specifically accumulated in response to *Xcm* race 18, reaching an optimum at 14 hpi (Figure 2B). Altogether, our data suggest a role for OPDA and JA in signaling events associated with resistance to *Xcm*.

The AtORA47 ortholog gene, *GhERF-IIB3*, is specifically up-regulated in HR-developing cotton cotyledons

The data described above prompted us to investigate molecular mechanisms responsible for the regulation of JA synthesis in pathogen-challenged cotton plants. Recent work pointed out the ERF TF OCTADECANOIC-RESPONSIVE ARABIDOPSIS AP2/ERF

DOMAIN 47 (AtORA47) as a positive regulator of *AtLOX3* and *AtAOC2* expression upon wounding and biotic stress in *A. thaliana* leaves (Walley *et al.*, 2007). Ethylene-response factor (ERF) proteins form a TF family mainly involved in stress responses and secondary metabolite production. Interestingly, it was shown that AtORA47 is able to directly interact with the two gene promoters, mediating their transcriptional trans-activation (Pauwels *et al.*, 2008; Zarei *et al.*, 2011). Although it was not experimentally established that AtORA47 promotes JA production in the latter works, we assumed that this TF and its relatives could do so and literally “switch on” the octadecanoic pathway in plants. We thus carried out a phylogenetic analysis of AtORA47 relative proteins and found that they formed a distinct group within the ERF family (Figure 3A), previously reported as group IIb (Champion *et al.*, 2009). Clustering strength of the group IIb was also supported by the presence of the conserved specific motifs of group II dubbed CMII-1 and CMII-2 (Figure 3B and (Nakano *et al.*, 2006)). Among the identified putative TFs, GhERF-IIb3 originating from the cotton plant *Gossypium hirsutum* exhibited an enhanced expression specific to *Xcm* race 18 infection characterized by a biphasic induction pattern (Figure 3C). In addition, the *GhERF-IIb3* gene, like its *A. thaliana* counterpart *AtORA47*, was responsive to JA whereas it did not respond to SA (Supplemental Figure 2 and (Pauwels *et al.*, 2008). Combined with data from Figure 2, this last set of results indicates that GhERF-IIb3 may be responsible for JA production during *Xcm*-induced HR by positively regulating octadecanoic pathway genes.

Nuclear GhERF-IIb3 proteins activate *AtAOC2* promoter in plant cells

Given that GhERF-IIb3 is a putative TF, it should present a typical nuclear localization. This hypothesis was challenged using a co-localization approach. The GhERF-IIb3 protein fused to GFP in its C-terminal end was co-expressed with the nuclear DsRed-tagged NtKIS1a protein in *A. thaliana* leaf protoplasts and imaged by fluorescence

microscopy. As shown in Figure 4A, both protein combinations exhibit a round shape fluorescent pattern consistent with a nuclear localization. In addition, GhERF-IIb3-associated fluorescence shows exact overlap with that of the nuclear marker NtKIS1 in good agreement with our working hypothesis.

The TF activity of GhERF-IIb3 was next tested using a promoter transactivation assay in *A. thaliana* leaf protoplasts. Protoplasts were cotransformed with a tripartite vector system that comprises (i) a reporter plasmid carrying the *AtAOC2* promoter-driven β -D-glucuronidase (*GUS*)-encoding *uidA* gene, (ii) an effector plasmid carrying *AtORA47*, *GhERF-IXa5* or *GhERF-IIb3* genes under the control of the *CaMV35S* promoter and (iii) a reference plasmid carrying the firefly luciferase gene. Relative enzymatic activity of the reporter protein was monitored *in vitro*, and normalized to constitutively expressed luciferase activity (Figure 4B).

While empty vector and expression of unrelated GhERF TF that belongs to the group IX (e.g. GhERF-IXa5) had minimal or no effect on GUS activity, *AtORA47* and, to a lesser extent, GhERF-IIb3 were clearly able to trans-activate the *AtAOC2* promoter in *A. thaliana* cells (Figure 4C). Similar results were also obtained when these two TFs were expressed in cotton protoplasts (Supplemental Figure 3), indicating that GhERF-IIb3 may share with *AtORA47* regulatory binding site(s) to trans-activate the *AtAOC2* promoter. Indeed, in the *A. thaliana* protoplast assay, the use of an *AtAOC2* promoter mutated in the GCC-like box responsible for *AtORA47* binding drastically diminished the GUS activity induced by both the *A. thaliana* and cotton proteins (Figure 4C).

Altogether, these data point GhERF-IIb3 and *AtORA47* as potent nuclear transcriptional activators of genuine JA biosynthesis genes in plant cells as well as a common mode-of-action for both TFs.

Overexpressing *GhERF-Iib3* TFs induces OPDA and JA accumulation in cotton cotyledons.

Whether transient expression of *GhERF-Iib3* in cotton cotyledons allowed for OPDA/JA accumulation was next examined. An efficient *Agrobacterium*-mediated expression system was successfully developed for this gain-of-function approach. Cotyledons of ten day-old cotton plants were transformed with *GhERF*-overexpressing constructs using as vector the *virG*-modified LBA1119 *Agrobacterium* strain (*virGN54D*), which improve T-DNA transfer frequency (Supplemental Figure 4; (Fits *et al.*, 2000)). Overexpression of the *GhERF-Iib3* gene resulted in substantial up-regulation of *GhAOS-like*, *GhAOC2-like* and *GhACX1a-like* expression (Figure 5A). This increase in gene expression was also observed when the *A. thaliana* AtORA47 TF-encoding gene was ectopically expressed in cotton cotyledons (Supplemental Figure 5A). Remarkably, the expression induction of the three putative JA biosynthesis genes previously identified by *GhERF-Iib3* and AtORA47 was accompanied by OPDA/JA accumulation in transformed tissues (Figure 5B and Supplemental Figure 5B), suggesting that the control of the octadecanoic pathway by AtORA47 and GhERF-Iib3 TFs could be conserved in higher plants.

Overexpressing *GhERF-Iib3* confers resistance to bacterial blight disease in cotton plants

Having established a system that permits *in planta* accumulation of OPDA/JA at the flick of GhERF-Iib3 TF switch, we next investigated the role of the hormone and its precursor in *Xcm* / cotton plant interactions. Two days following agroinfiltration, cotton cotyledons overexpressing *GhERF-Iib3* were inoculated with the virulent (race 20) bacterial strain. Surprisingly, while control leaves expressing GFP6 showed typical disease symptoms characterized by spreading yellowing zones, *GhERF-Iib3*-transformed tissues presented HR-

like lesions reminiscent of the resistance phenotype (Figure 6A). At 12 dpi, *GhERF-IIb3*-induced hypersensitive-like cell death resulted in 60% water loss (Figure 6B) associated with a strong decrease in foliar bacterial concentrations that was not observed in control tissues expressing the GFP6 (Figure 6C). Consistent with increased OPDA/JA levels in cotyledons, this phenotype was accompanied by the up-regulation of *GhAOS-like*, *GhAOC2-like* and *GhACX1a-like* genes, as well as that of additional genes responsive to JA which include two TF *GhERF-IXa1* and *GhERF-IXa2* and the lipoxygenase *GhLOX1*, a hallmark of cotton hypersensitive cell death (Supplemental Figure 6). Likewise, ectopic expression of *AtORA47* in cotton cotyledons also phenocopied the resistance response following virulent strain (race 20) inoculation that normally triggers disease (Supplemental Figure 7).

Altogether, these results point to a positive control of hypersensitive cell death by *GhERF-IIb3* and OPDA/JA in cotton plants challenged with *Xcm*.

DISCUSSION

To date, molecular mechanisms that control JA production remain poorly understood. Here, we have identified the TF, *GhERF-IIb3*, as a novel positive regulator of JA synthesis in cotton. In this work, we have established that (i) *GhERF-IIb3* is rapidly and transiently up-regulated in response to the avirulent *Xcm* strain (Figure 3C), (ii) this change in gene expression takes place before the activation of the three identified JA biosynthesis gene candidates and raise in oxylipin metabolite concentrations (Figure 2A and 2B), and (iii) overexpressing *GhERF-IIb3* leads to OPDA and JA over accumulation in uninfected cotton cotyledons (Figure 5B). In addition to a role for JA and its precursor in defense mechanisms associated with cotton HR, these data suggest that *GhERF-IIb3* proteins could activate the octadecanoic pathway, in a linear model where a yet-to-be characterized *Xcm*-generated signal induces the TF expression, causing OPDA and JA to accumulate. In line with this

model, it is expected that genuine octadecanoic genes whose expression is driven by GCC-like motif-containing promoters would be directly regulated by the GhERF-IIb3 TF, as pointed by the transactivation promoter assay (Figure 4). It cannot be ruled out, nonetheless, that OPDA could be released from arabidopside-like lipids sustaining part of the *Xcm*-dependent JA production. Moreover, given that *GhAOS-like*, *GhAOC2-like*, *GhACX1a-like* and *GhERF-IIb3* genes are responsive to exogenous application of JA, it is tempting to speculate that the ERF TF also contributes to the positive feedback amplification loop that operates in JA signaling, though this assumption would require further investigation to be proved.

Remarkably, we have also demonstrated that overexpressing *GhERF-IIb3* in cotton cotyledons converts a compatible interaction into an incompatible one (Figure 6). In other words, plants, which are normally susceptible to the virulent race 20 of *Xcm*, become resistant to the bacterium when they accumulate OPDA and JA. This resistant phenotype presents the two main characteristics of HR: cell death lesions localized to the infection site and low foliar bacterial growth a few days post-inoculation. An executioner of cotton HR-associated cell death, namely *GhLOX1* (Jalloul *et al.*, 2002; Marmey *et al.*, 2007), is also transcriptionally up-regulated in this context (Supplemental Figure 6). Altogether, these results strongly suggest that the increase in OPDA/JA levels recorded in response to the avirulent race 18 of *Xcm* could be responsible for hypersensitive cell death initiation within the infected zone by activating the 9-LOX-encoding gene *GhLOX1*. As proposed earlier (Jalloul *et al.*, 2002), the GhLOX1-dependent lipid peroxidation could contribute to cellular structure dismantling during cotton HR. Our previous work lends support to this scenario. We have indeed showed that (i) MeJA-pretreated cotton cotyledons exhibit a strong accumulation of 9-LOX activity accompanied by *GhLOX1* gene expression, and (ii) infiltration of the pretreated cotyledons with LOX substrates, i.e. polyunsaturated fatty acids (PUFA), triggers hypersensitive-like

symptoms in the absence of pathogens (Marmey *et al.*, 2007). Hence, overexpression of the *GhERF-Iib3* TF could prime HR through constitutive activation of the 9-LOX gene, allowing hypersensitive cell death to be launched in the presence of the compatible race 20 of *Xcm*. If so, this situation implies the onset of lipid-hydrolyzing activities acting upstream of GhLOX1, since 9-LOX preferentially oxidize free PUFA. The patatin-like protein GhPat1 was previously suggested to form an enzymatic duo with GhLOX1 during cotton immunity and represents therefore a good candidate for releasing the substrates of the 9-LOX (Cacas *et al.*, 2009).

In the literature, contradictory results have been published with regards to the function of JA in controlling programmed cell death (PCD). On the one hand, MeJA treatment of WT *A. thaliana* plants was reported to inhibit ozone-induced cell death, a process which shares features with hypersensitive cell death (Tuominen *et al.*, 2004). One such hormonal treatment also restricts lesion spreading in the ozone-sensitive mutant *radical-induced cell death1* (Overmyer *et al.*, 2000). On the other hand, the development of HR-like symptoms triggered by the mycotoxin fumonisin B1 requires JA to develop (Asai *et al.*, 2000). Likewise, JA promotes singlet oxygen-dependent cell death in *flu* mutants altered for chlorophyll anabolism (Danon *et al.*, 2005). Furthermore, OPDA was genetically proven to repress foliar necrotic lesions in *flu* mutants. In the cotton/*Xcm* patho-system it is difficult to conclude on the respective roles of OPDA and JA in cell death propagation at the edge of infected areas and in naïve systemic tissues, since both molecules accumulate upon *GhERF-Iib3* expression. Nonetheless, our results clearly establish that octadecanoic pathway products are invoked following biotrophic bacteria attack. This unexpectedly extends previous works that demonstrated synergistic effects for ethylene and JA in the onset of defense against necrotrophic fungi, as well as antagonism between SA and JA in response to the biotrophic pathogen *Pseudomonas syringae* in *A. thaliana* (Pieterse *et al.*, 2012).

Beyond academic research, controlling *in situ* JA production represents a major challenge not only to restrict pest infection in the field, but also for many additional horticultural and biotechnological applications (Wasternack, 2014). However, despite the fact that several JA regulatory components have been identified as aforementioned, there is currently no experimental system that allows to tightly controlling JA production at the spatio-temporal levels. In addition, manipulating the rate-limiting step of the octadecanoic pathway is not as straightforward as expected. The failure to significantly increase endogenous JA levels when *AOS* and *AOC* genes were overexpressed in distinct plant species illustrates the difficulty of this task (Laudert *et al.*, 2000); (Stenzel *et al.*, 2003). Here, we report on a novel alternative that can be potentially used for manipulating the octadecanoic pathway *in situ*. Based on sequence similarities and phylogenetic analysis (Figure 3A and 3B and Supplemental Table 3), many plant models including monocotyledonous plants like rice (*Oryza sativa*) and dicotyledonous plants like tobacco (*N. Benthamiana*), alfalfa (*Medicago truncatula*), tomato (*Solanum lycopersicum*) and grapevine (*Vitis vinifera*) contain AtORA47 sequence orthologs unrooted in the group IIb, suggesting that expressing *GhERF-IIb3*, *AtORA47* or endogenous AtORA47-related TF in all these plant species could allow to “switch on” JA synthesis on demand.

EXPERIMENTAL PROCEDURES

Biological materials, plant infection and treatments.

Cotton cultivar *Gossypium hirsutum* cv. Reba B50, which carries *B2B3* blight-resistance genes, was used in the present study. This cultivar is resistant to race 18 of the bacterium *Xanthomonas citri* pv. *malvacearum* (*Xcm18*), but develops disease symptoms in response to race 20 (*Xcm20*) (Innes, 1983). Plants were grown and infected with *Xcm* strains as previously described (Jalloul *et al.*, 2002). *Xcm18*-triggered hypersensitive cell death

symptoms were visually assessed and estimated by quantifying water loss (Rustérucci *et al.*, 1999). Bacterial counting assays were performed at 1, 6 and 12 days post-inoculation of *Xcm20* strain. Three distinct sets of serial dilutions were made for 9 infected cotyledons. Twelve-mm diameter-discs were punched out from inoculated cotyledons, disinfected with 70% ethanol for 2 min, rinsed twice and ground in 5 mL sterile water. The resulting suspension was serially diluted in water, and 100 μ L of dilutions were plated on LPG agar plates before being incubated at 28°C until colony counting. Cotyledon from twelve-day old cotton plants were wounded 4 times with a hemostat. Damaged cotyledons were harvested in liquid nitrogen after wounding for real-time PCR (qRT-PCR) and HPLC-MS. *A. thaliana thaliana* accession Columbia-0 and *Gossypium hirsutum* cv. Reba B50 were used to generate protoplasts from rosette leave and cotyledons respectively (Yoo *et al.*, 2007).

Sequence analysis.

GhERF and coding sequences of JA biosynthesis gene homologs were retrieved from TIGR cotton database (<http://plantta.tigr.org/search.shtml>) using TBLastN tool, as described in (Champion *et al.*, 2009). Amino acid sequence alignments and phylogenetic analysis were performed using phylogeny.fr with default settings. The tree was edited with FigureTree (<http://tree.bio.ed.ac.uk/software/Figuretree/>).

Gene expression analysis.

Transcript levels of cotton JA-biosynthesis gene candidates, *GhLOX1*, the *GhERF-Iib3* and *GhERF-IXa* groups were quantified by qRT-PCR with MX3005P (Stratagene, France) as described by (Champion *et al.*, 2009). Relative expression levels of reporter and target genes were determined based on the 2- $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001)

using cotton *Actin2* (AY305724) as internal control (Champion *et al.*, 2009)(Li *et al.*, 2005).

Primers for qRT-PCR analysis are listed in Supplemental Table 2.

Plasmid constructs.

Cotton plants were transiently transformed with Gateway binary vector pMDC32 (for overexpression) (Curtis and Grossniklaus, 2003) by agroinfiltration. Reverse primers were designed to amplify cDNA with or without stop codon for each coding sequences *AtORA47* (At1g74930) and *GhERF-Iib3* (accession number KJ944170) from *A. thaliana* or cotton plants treated by JA during 1 h (see Supplemental Table 2 for primer sequences). As control, *GFP6* was amplified using pMDC84 as template in PCR reaction. All PCR products were first cloned into pENTR-D TOPO (Invitrogen), and then were recombined into the binary plasmids pMDC32 or pMDC43 using LR Clonase (Invitrogen). Plasmid constructs were confirmed by sequencing. Plasmids were electroporated into *Agrobacterium tumefaciens* LBA1119m containing *pBBRvirGN54D* plasmid and used for cotton cotyledon transformation.

For the trans-activation promoter assay (see dedicated Methods' section below), *A. thaliana* and cotton protoplasts were co-transformed with plasmids carrying *AtORA47*, *GhERF-IXa5* (accession number KJ944171) or *GhERF-Iib3* and the reference plasmids p2rL7 carrying the *Renilla reniformis* luciferase gene and a version of the *AtAOC2* promoter-*uidA* fusion. *A. thaliana* genomic DNA was used as template for amplifying a 323 bp-long fragment of the *AtAOC2* promoter using specific primers listed in Supplemental Table 2. The mutated promoter derivative pAOC2m was generated using a mutated version of the primer used to amplify the wild type sequence of pAOC2 (see Supplemental Table 2 for primer sequence). PCR products were cloned into pENTR-D TOPO (Invitrogen), and were recombined into pMDC162 (Curtis and Grossniklaus, 2003).

Plant transformation.

Agrobacterium-mediated transient expression for cotton cotyledon were performed as described (Sparkes *et al.*, 2006). *Agrobacterium tumefaciens* strains were grown for 18 h at 29°C under mild shaking. *Agrobacteria* density was estimated spectrophotometrically at 600 nm. When in the exponential phase (between 0.6 and 0.8 OD), bacteria were sedimented (3000 g for 20 minutes at 4°C) and the pellet was resuspended in the infiltration medium (10 mM MgSO₄, 200 μM Acetosyringone and 20 mM MES pH 5.5) to reach a final OD of 0.5. Agroinfiltration was carried out on 10-old-day cotton cotyledons. Each cotyledon was inoculated using a needle-less syringe.

Quantification of OPDA and JA contents.

Leaf samples harvested from cotton plants were ground with mortar and pestle in liquid nitrogen. The resulting powder (250 mg/sample) was extracted with 1 mL methanol containing 100 ng deuterated jasmonic acid (d6Ja) and 100 ng deuterated OPDA (d5OPDA), as internal standards. Methanolic extracts were centrifuged at 5000 g for 10 min at 4°C and the supernatant was recovered in glass tubes. Pellets were reextracted 3 times, methanolic extracts were pooled and dried at 40°C under nitrogen flux. Dried residues were resuspended in 5 mL of 100 mM phosphate buffer pH 7.8, containing 5% (w/v) NaCl. Suspensions were then washed three times with 2.5 mL hexane and organic phases were discarded. The pH of the clean pooled aqueous phases was adjusted to 1.4 using 5N HCl. Aqueous phases were extracted three times using 2.5 mL chloroform. Pooled chloroform phases were finally dried at 40°C under nitrogen flux and dry residues stored at -80°C until use. Samples were resuspended before LC-MS analysis in 100 μL methanol (injected volume: 20μL).

Dosage of JA and OPDA was performed using a Waters 1515 liquid chromatography (LC) coupled to a Waters ZQ mass spectrometer (MS) controlled by the software MassLynk V5.0.5 (MicroMass, Cray, USA). The LC column used was an XTerra MS C18, 3.5 μ m, 100 x 2.1 mm (Waters, Milford, MA, USA) and gradient elution was performed. A binary solvent was utilized, consisting of water with 15 mM formic acid (eluent A) and methanol (eluent B). The initial conditions were 40% A at a flow rate of 0.25 mL/min and kept constant for 2 min. A gradient elution was introduced to change the chromatographic conditions to 60% A at 14 min to flush the analytical column, followed by restoring the column to the initial conditions at 16 min followed by equilibrium for 2 min. Under these conditions, the retention time of JA and its deuterated form was 9.3 min, while the retention of OPDA and its deuterated form was 16.5 min. Both JA and OPDA were detected and quantified in negative ESI (Electron Spray Ionisation) mode. The analysis conditions for mass spectrometry were as follows: vaporization temperature at 120°C, source temperature at 450°C, Capillary voltage 2.5 KV, Cone voltage 20 V. The quantitation was carried out in the “selected ion monitoring” mode using characteristic ions: 209 m/z ion for JA, 215 m/z ion for deuterated JA, 291 m/z ion for OPDA, and 295 m/z ion for deuterated OPDA. The amounts of endogenous compounds were calculated from the signal ratios of the unlabelled over the stable isotope-containing mass fragments observed in both analyzing channels.

Protoplast transfection, reporter enzyme assays and fluorescence microscopy.

The protoplasts of cotton cotyledons and *A. thaliana* leaves were produced and transformed using a protocol from (Yoo *et al.*, 2007). Co-transformations of pAOC2- *uidA* or pAOC2m- *uidA* with the both p2rL7 plasmid and one of the effector plasmids carrying *AtORA47*, *GhERF-IXa5*, or *GhERF-IIb3*, were carried out. Empty pMDC32 expression vector was used as an effector control. Protoplasts were transformed using polyethylene glycol as

described previously (Yoo *et al.*, 2007) with the three constructs in a ratio of 2:2:6 (GUS:LUC:effector plasmid). The protoplasts were collected 18 hours after transformation and frozen in liquid nitrogen. GUS and LUC activities were measured as previously described (Zarei *et al.*, 2011). In each sample, GUS and LUC activities were taken into account to correct the differences in efficacy of transformation and of protein extraction. The mean ratio GUS/LUC was expressed relatively to the control empty vector.

In order to determine the subcellular localization of the GhERF-IIb3 TF, protoplasts were co-transformed with the plasmids allowing the expression of GFP6-tagged candidate proteins and that of the *NtKIS1a-DsRFP* (Jasinski, 2002), and then incubated in the dark for at least 16 hours at 25°C. Fluorescence patterns were examined by fluorescence microscopy (Zeiss Axio Imager A1 epifluorescence microscope coupled to a Colibri LED illumination system from Zeiss and a Retiga-SRV from QImaging CCD camera).

Statistics.

Data collected from protoplast transactivation experiments were subjected to a one-way analysis of variance (ANOVA) in order to determine if there were significant differences in *AtAOC2* promoter activity between treatments. Means were compared with Tukey post-hoc tests for pairwise comparisons ($p < 0.01$).

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The INRA and IRD institutes has filed a patent on behalf of inventors P.D., M.N. and A.C. on the use of AtORA47 to switch on overproduction of jasmonic acid in transgenic plants and an improved resistance to pathogenic agents.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION LEGENDS

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FIGURE LEGENDS

Figure 1. Wounding promotes both up-regulation of JA biosynthesis-related gene expression and OPDA/JA accumulation in cotton cotyledons. (A) Twelve-day old cotton plants were wounded 4 times with a hemostat. Damaged cotyledons were harvested at the indicated time-points after wounding. OPDA/JA levels were determined by LC-MS. (B) Expression of cotton *ALLENE OXIDE SYNTHASE-like* (*GhAOS-like*), *ALLENE OXIDE CYCLASE 2-like* (*GhAOC2-like*) and *ACYL-COENZYME A OXIDASE-like 1a* genes (*GhACX1a-like*) in damaged cotyledons was quantified by qRT-PCR. Results in (A) and (B) are averages of three biological replicates, error bars indicate standard deviation (n>12).

Figure 2. Coordinated induction of the JA biosynthesis-related gene expression and metabolite accumulation upon challenge with the avirulent strain of *Xcm*. (A) Time course expression of JA biosynthesis-related genes *GhAOS-like*, *GhAOC2-like* and *GhACX1a-like* in response to *Xanthomonas* virulent (*Xcm20*) and avirulent (*Xcm18*) strains. Gene expression was measured by q-RT-PCR. Results are averages, error bars indicate SD

(n>12). (B) Time course of OPDA and JA production in response to *Xcm* infection. Results are averages, error bars indicate SD (n>12). Asterisks denote values significantly different from the control (Student test, * for p<0.05; ** for p<0.01). All these experiments (A and B) were repeated at least three times with similar results.

Figure 3. Identification of an ERF-related transcription factor induced during *Xcm*-induced HR in cotton plants. (A) A maximum likelihood tree representing relationships among ERF proteins from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Medicago truncatula* (Mt), *Solanum lycopersicum* (Sly), *Nicotiana tobacco* (Nt), *Glycine max* (Gm), *Vitis vinifera* (Vv), *Populus trichocarpa* (Pt), *Broussonetia papyrifera* (Bp), *Physcomitrella patens* (Pp) and *Gossypium hirsutum* (Gh) plants is rooted to APETALA2 AP2-domain R1 (At4g36920). Bootstrap values from 100 replicates were used to assess branch support. Bootstrap values over 70 are shown. The classification by (Nakano *et al.*, 2006) is indicated by colored accession numbers. (B) Alignment of the *Gossypium hirsutum* GhERF-IIb3 with the *Arabidopsis* ERF transcription factor AtORA47. Underlined sequences represent the AP2-domain and the two ERF group Iib-specific conserved motifs CMII-1 and CMII-3. Strictly conserved residues are highlighted in black. Numbers on the right of the alignment indicate amino acid position for ERF transcription factors. (C) Expression levels of *GhERF-IIb3* in response to *Xanthomonas* virulent (*Xcm20*) and avirulent (*Xcm18*) strains, measured by qRT-PCR. Results are averages, error bars indicate SD (n>12). These experiments were repeated at least three times with similar results.

Figure 4. Transient overexpression of *Xcm*-inducible nuclear transcription factors GhERF-IIb3 trans-activate the *AtAOC2* promoter in *A. thaliana* cells.

(A) Subcellular localization of the TF GFP fusions GhERF-IIb3-GFP in *A. thaliana* leaf protoplasts. GhERF-IIb3-GFP and NtKISa-DsRED constructs were transformed simultaneously in protoplasts. The differential interference contrast (DIC) images are shown at the left, epifluorescence microscopic images are shown in the middle and the merged images are at the right. (B) Schematic representation of constructs used for promoter transactivation assays in *A. thaliana* protoplasts. The reporter construct consisted of the *uidA* gene driven by *AtAOC2* promoter (or *AtAOC2* mutated promoter (*pmAtAOC2*) as described by (Zarei *et al.*, 2011). The effector constructs consisted of an expression vector carrying the *CaMV35S* promoter upstream of the *GhERF-IIb3*, *AtORA47* or *GhERF-IXa5* cDNAs. Empty effector vector was used as a negative control. The Firefly luciferase (*LUC*) gene fused to the *CaMV35S* promoter served as a reference gene to correct for differences in transformation and protein extraction efficiencies. Firefly luciferase coding sequence (*ffLUC*) driven by the promoter *CaMV35S* was used as internal control. (C) *AtAOC2* promoter trans-activation by the transcription factors GhERF-IIb3 and AtORA47 in plant protoplasts. Bars represent average GUS/LUC ratios from triplicate experiments (\pm SD) expressed relative to the vector control set at 100% (n= 6). Letters above the bars indicate distinct statistical groups as calculated by one-way ANOVA (P<0.01).

Figure 5. Overexpression of *GhERF-IIb3* promotes OPDA and JA accumulation in cotton plants. (A) Activation of JA-responsive marker gene expression in cotton cotyledons overexpressing *GhERF-IIb3*. (B) OPDA and JA contents in cotton cotyledons overexpressing *GhERF-IIb3*. Ten day-old *G. hirsutum* plants were transformed with *Agrobacterium tumefaciens* carrying *GhERF-IIb3* or *GFP6*. Two days post-transformation, gene expression and oxylipin content were determined by qRT-PCR and LC-MS, respectively. Averages \pm SD (n=24) were calculated from biological triplicates.

Figure 6. Overexpression of *GhERGF-Iib3* induces bacterial blight resistance in cotton plants.

(A) Cotton cotyledons overexpressing *GhERF-Iib3* or *GFP6* as control were inoculated at 10^8 CFU/mL with the virulent *Xcm* strain (race20). Symptom photographs at 12 days post-inoculation (dpi). Note the HR-like symptoms in *GhERFIib3*-expressing cotyledons compared to typical yellow disease symptoms in *GFP6* controls. (B) Cell death intensity at 12 dpi using water loss as proxy. (C) Kinetic evolution of cotyledon bacterial content. All experiments (B, C and D) were repeated at least three times with similar results. Averages \pm SD (n=12) were calculated from biological triplicates.

SUPPLEMENTARY DATA

Supplemental Figure 1. Genes encoding potential enzymes of jasmonate biosynthesis expressed in response to hormonal treatments in cotton.

(A) Schematic overview of the octadecanoid pathway leading to JA formation. (B) Expression of *GhAOS-like*, *GhAOC2-like* and *GhACX1a-like* in response to JA and salicylic acid (SA) treatments, measured by qRT-PCR. Twelve-day-old cotton plants were treated for two hours with 50 mM JA, 50 mM SA or the combination of solvents dimethyl sulfoxide (DMSO; 0.1%) and sodium phosphate, pH 7 (0.5 mM) as control (C). Results are averages of three biological replicates, error bars indicate standard deviation (n>12).

Supplemental Figure 2. Expression analysis of *GhERF-Iib3* genes in response to solvent

(C, control), JA and SA treatments. This experiment has been carried out as described in the Supplemental Figure 1.

Supplemental Figure 3. Both transcription factors AtORA47 and GhERF-IIb3 trans-activate the *AtAOC2* promoter in cotton protoplasts. Protoplasts were produced and transformed using a protocol adapted from Yoo et al. (2007). GUS and LUC activity tests were performed as previously described (Zarei et al., 2011). Averaged GUS/LUC ratios relatively expressed to the control vector. Three independent experiments \pm SD (n=6).

Supplemental Figure 4: Optimization of T-DNA transfer to cotton cotyledons cells using *Agrobacterium tumefaciens* LBA1119 *virG*^{N54D} bacterial as vector. (A) Comparison of transformation efficiency over time in agroinfiltrated cotyledons using two distinct bacterial strains. *A. tumefaciens* strains LBA1119 and LBA1119 *virG*^{N54D} were both transformed with the *pCAMBIA1300::uidA-intron* and further used for the transformation of 10-day-old cotton cotyledons as described in the section *Material and Methods*. The *virG*-modified LBA1119 was chosen because it is known to improve T-DNA transfer frequency (Fits *et al.*, 2000). β -glucuronidase (GUS) activity was followed for 2 days for both strains. For each strains, 24 cotyledons were agroinfiltrated. GUS activities are reported as mean \pm SD. Experiments were repeated at least three times with similar results. (B) Quantitative analysis of β -GUS activity until 6 days post-infiltration with the LBA1119 *virG*^{N54D}. Twenty four cotyledons were agroinfiltrated. GUS activity is reported as mean \pm SD. The experiment was repeated two times with similar results. (C) Histochemical analysis of agro-infiltrated cotton cotyledons. Left half of the cotyledon was infiltrated with the empty bacterial strain as control whereas the right half was infiltrated using the *pCAMBIA1300-uidA-intron* plasmid-containing the *virG*-modified LBA1119 strain. The photograph (2 dpi) shows the presence of indigo crystals over the whole region that had been transformed by *pCAMBIA1300-GUS-intron*.

Supplemental Figure 5. Overexpression of *AtORA47* increases gene expression of JA-biosynthesis marker genes and endogenous OPDA/JA levels in cotton. (A) Expression of *GhAOS-like*, *GhAOC2-like* and *GhACX1a-like* genes in cotton cotyledons overexpressing *AtORA47* or GUS-encoding *uidA* genes. (B) OPDA and JA contents in cotton cotyledons overexpressing *AtORA47* or GUS-encoding *uidA* genes. Ten day-old-*G. hirsutum* plants were transformed with *A. tumefaciens* carrying either the *AtORA47* or *uidA* constructs. Two days post-transformation, gene expression and oxylipin content were determined by qRT-PCR and LC-MS, respectively. Averages \pm SD (n=12) were calculated from biological triplicates.

Supplemental Figure 6. Gene expression of JA-responsive and defense marker genes in transgenic cotton overexpressing *GhERF-IIb3* and inoculated by the virulent *Xcm20* strain. Relative transcript levels were determined by qRT-PCR at one day post-inoculation with *Xcm20* in control tissues expressing GFP6- and in *GhERF-IIb3*-overexpressing tissues. Means \pm SD (n=12). Experiments were repeated at least two times with similar results.

Supplemental Figure 7. Transient ectopic expression of *AtORA47* in cotton cotyledons confers resistance to virulent *Xcm* (race 20) bacteria. Cotton cotyledons overexpressing *AtORA47* or *GFP6* were inoculated at 10^8 CFU/mL and bacterial content was determined over time as described in the section Material and Methods. Means \pm SD (n=12). Experiments were repeated two times with similar results.

Supplemental Table 1. JA biosynthesis gene candidates *AOS*, *AOC* and *ACX* from *Gossypium hirsutum* share a significant degree of sequence conservation with their *Arabidopsis thaliana* counterparts.

Supplemental Table 2. List of primers used in this study.

Supplemental Table 3. List of AP2-domains used in phylogenetic analysis.

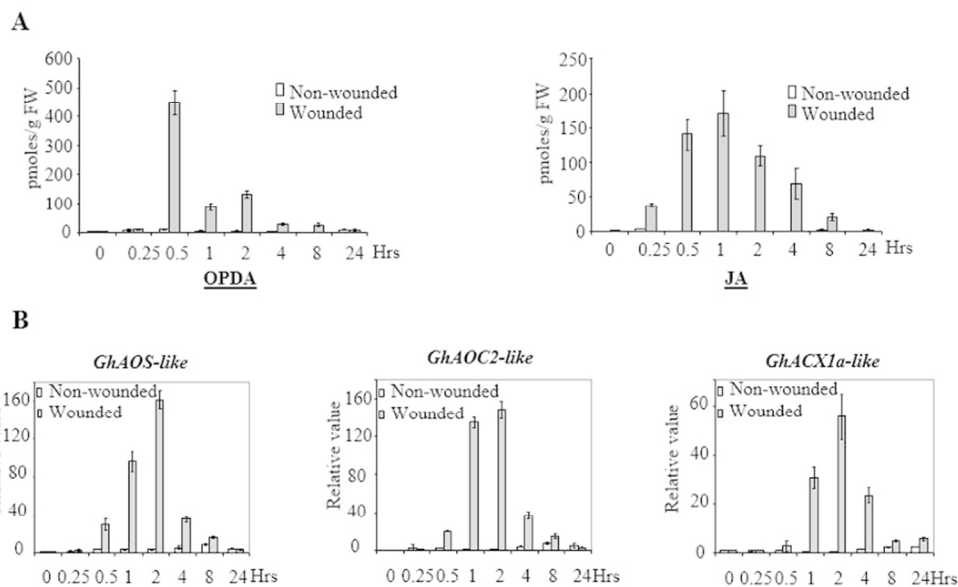


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(B) Expression of cotton *ALLENE OXIDE SYNTHASE-like* (*GhAOS-like*), *ALLENE OXIDE CYCLASE 2-like* (*GhAOC2-like*) and *ACYL-COENZYME A OXIDASE-like 1a* genes (*GhACX1a-like*) in damaged cotyledons was quantified by qRT-PCR.

Results in (A) and (B) are averages of three biological replicates, error bars indicate standard deviation ($n > 12$).

- Figure 1. Wounding promotes both up-regulation of JA biosynthesis-related gene expression and OPDA/JA accumulation in cotton cotyledons. (A) Twelve-day old cotton plants were wounded 4 times with a hemostat. Damaged cotyledons were harvested at the indicated time-points after wounding. OPDA/JA levels were determined by LC-MS. (B) Expression of cotton *ALLENE OXIDE SYNTHASE-like* (*GhAOS-like*), *ALLENE OXIDE CYCLASE 2-like* (*GhAOC2-like*) and *ACYL-COENZYME A OXIDASE-like 1a* genes (*GhACX1a-like*) in damaged cotyledons was quantified by qRT-PCR. Results in (A) and (B) are averages of three biological replicates, error bars indicate standard deviation ($n > 12$).

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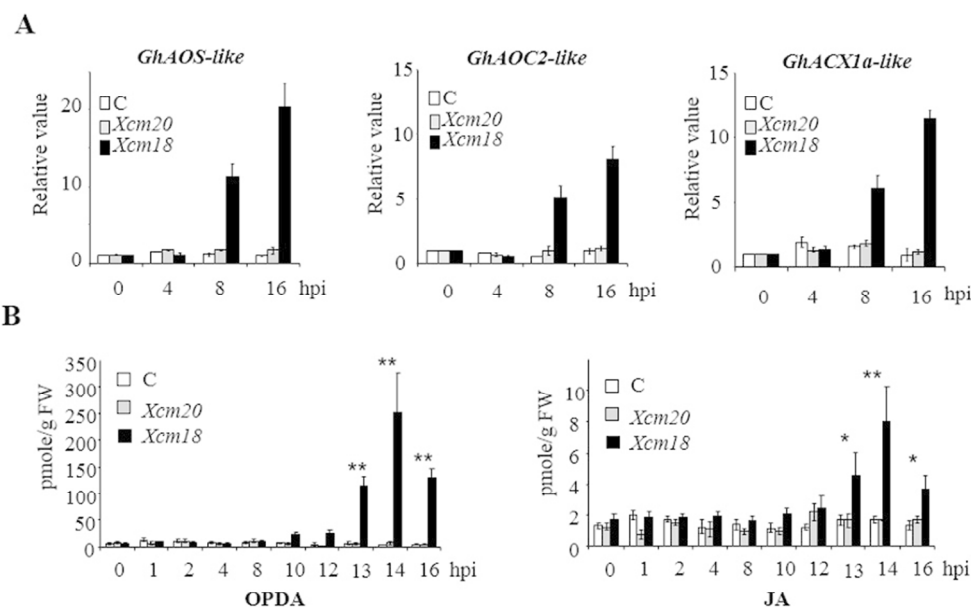


Figure 2. Coordinated induction of the JA biosynthesis-related gene expression and metabolite accumulation upon challenge with the avirulent strain of *Xcm*.

(A) Time course expression of JA biosynthesis-related genes *GhAOS-like*, *GhAOC2-like* and *GhACX1a-like* in response to *Xanthomonas* virulent (*Xcm20*) and avirulent (*Xcm18*) strains. Gene expression was measured by q-RT-PCR. Results are averages, error bars indicate SD (n>12).

(B) Time course of OPDA and JA production in response to *Xcm* infection. Results are averages, error bars indicate SD (n>12). Asterisks denote values significantly different from the control (Student test, * for p<0.05; ** for p<0.01).

All these experiments (A and B) were repeated at least three times with similar results.

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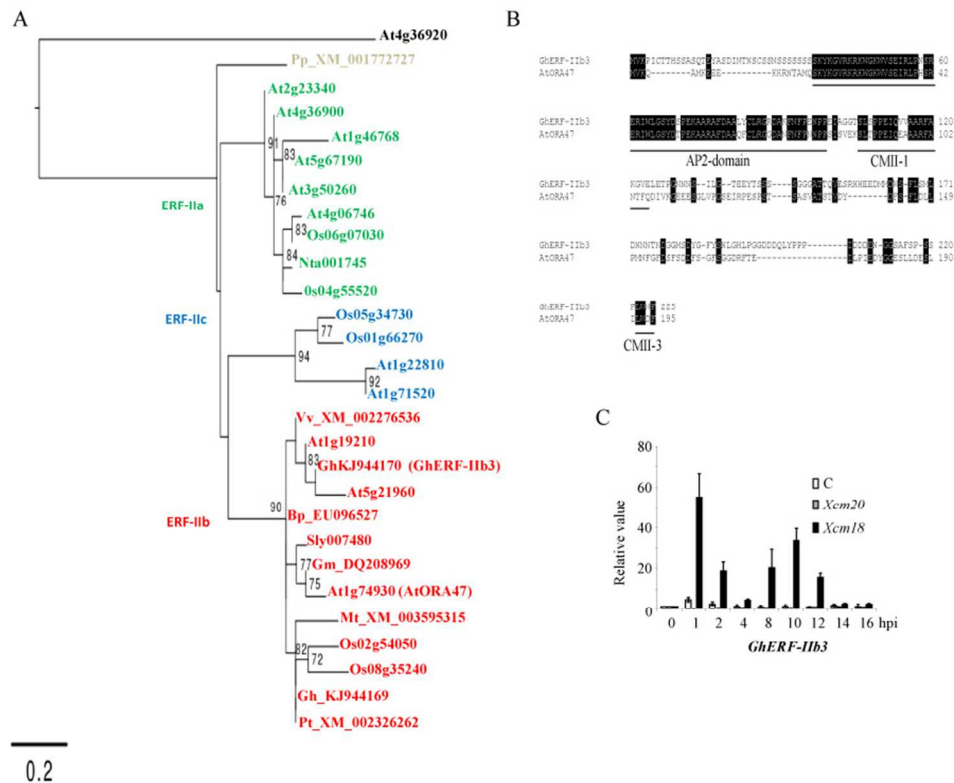
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Figure 3. Identification of an ERF-related transcription factor induced during *Xcm*-induced HR in cotton plants.

(A) A maximum likelihood tree representing relationships among ERF proteins from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Medicago truncatula* (Mt), *Solanum lycopersicum* (Sly), *Nicotiana tobacco* (Nt), *Glycine max* (Gm), *Vitis vinifera* (Vv), *Populus trichocarpa* (Pt), *Broussonetia papyrifera* (Bp), *Physcomitrella patens* (Pp) and *Gossypium hirsutum* (Gh) plants is rooted to APETALA2 AP2-domain R1 (At4g36920). Bootstrap values from 100 replicates were used to assess branch support. Bootstrap values over 70 are shown. The classification by ³² is indicated by colored accession numbers.

(B) Alignment of the *Gossypium hirsutum* GhERF-IIb3 with the *Arabidopsis* ERF transcription factor AtORA47. Underlined sequences represent the AP2-domain and the two ERF group IIb-specific conserved motifs CMII-1 and CMII-3. Strictly conserved residues are highlighted in black. Numbers on the right of the alignment indicate amino acid position for ERF transcription factors.

(C) Expression levels of GhERF-IIb3 in response to *Xanthomonas virulent* (*Xcm20*) and avirulent (*Xcm18*) strains, measured by qRT-PCR. Results are averages, error bars indicate SD (n>12). These experiments were repeated at least three times with similar results.

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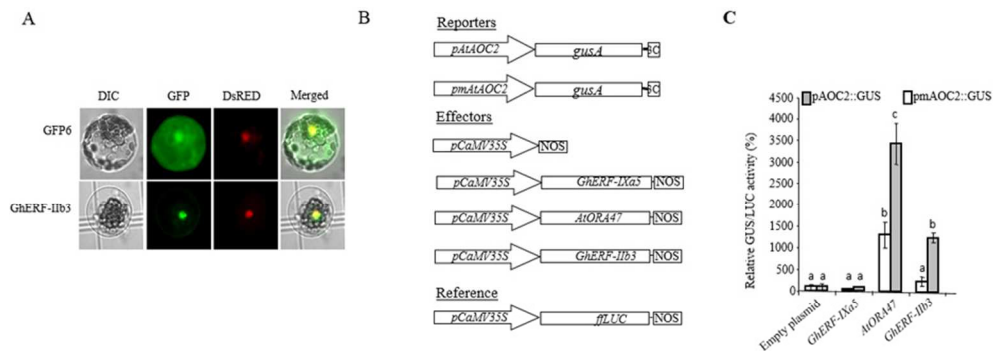


Figure 4. Transient overexpression of *Xcm*-inducible nuclear transcription factors GhERF-IIb3 trans-activate the *AtAOC2* promoter in *A. thaliana* cells. (A) Subcellular localization of the TF GFP fusions GhERF-IIb3-GFP in *A. thaliana* leaf protoplasts. GhERF-IIb3-GFP and NtKISA-DsRED constructs were transformed simultaneously in protoplasts. The differential interference contrast (DIC) images are shown at the left, epifluorescence microscopic images are shown in the middle and the merged images are at the right. (B) Schematic representation of constructs used for promoter transactivation assays in *A. thaliana* protoplasts. The reporter construct consisted of the *uidA* gene driven by *AtAOC2* promoter (or *AtAOC2* mutated promoter (*pmAtAOC2*)) as described by ³¹. The effector constructs consisted of an expression vector carrying the *CaMV35S* promoter upstream of the *GhERF-IIb3*, *AtORA47* or *GhERF-IXa5* cDNAs. Empty effector vector was used as a negative control. The Firefly luciferase (*LUC*) gene fused to the *CaMV35S* promoter served as a reference gene to correct for differences in transformation and protein extraction efficiencies. Firefly luciferase coding sequence (*ffLUC*) driven by the promoter *CaMV35S* was used as internal control. (C) *AtAOC2* promoter trans-activation by the transcription factors GhERF-IIb3 and AtORA47 in plant protoplasts. Bars represent average GUS/LUC ratios from triplicate experiments (\pm SD) expressed relative to the vector control set at 100% ($n = 6$). Letters above the bars indicate distinct statistical groups as calculated by one-way ANOVA ($P < 0.01$).

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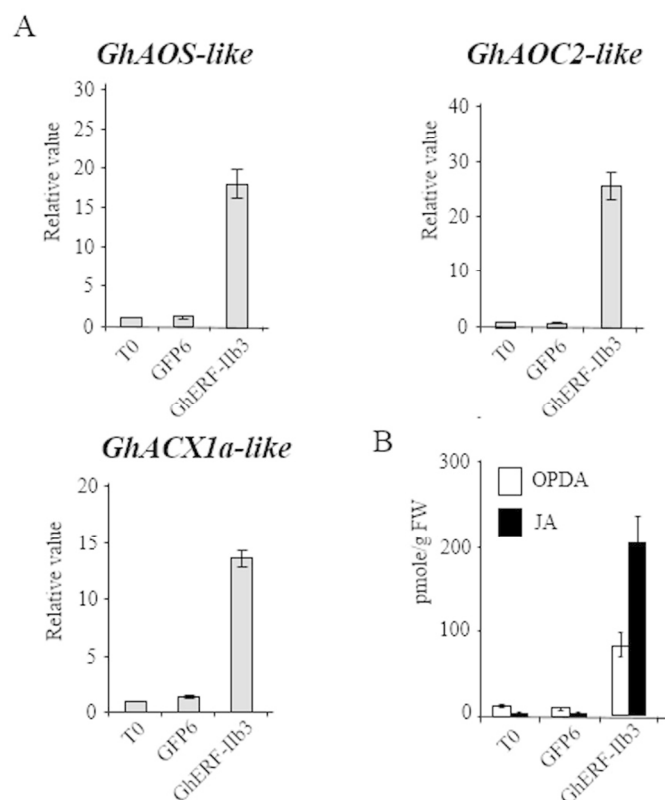


Figure 5. Overexpression of *GhERF-IIb3* promotes OPDA and JA accumulation in cotton plants.

(A) Activation of JA-responsive marker gene expression in cotton cotyledons overexpressing *GhERF-IIb3*.

(B) OPDA and JA contents in cotton cotyledons overexpressing *GhERF-IIb3*. Ten day-old *G. hirsutum* plants were transformed with *Agrobacterium tumefaciens* carrying *GhERF-IIb3* or *GFP6*. Two days post-transformation, gene expression and oxylipin content were determined by qRT-PCR and LC-MS, respectively. Averages \pm SD (n=24) were calculated from biological triplicates.

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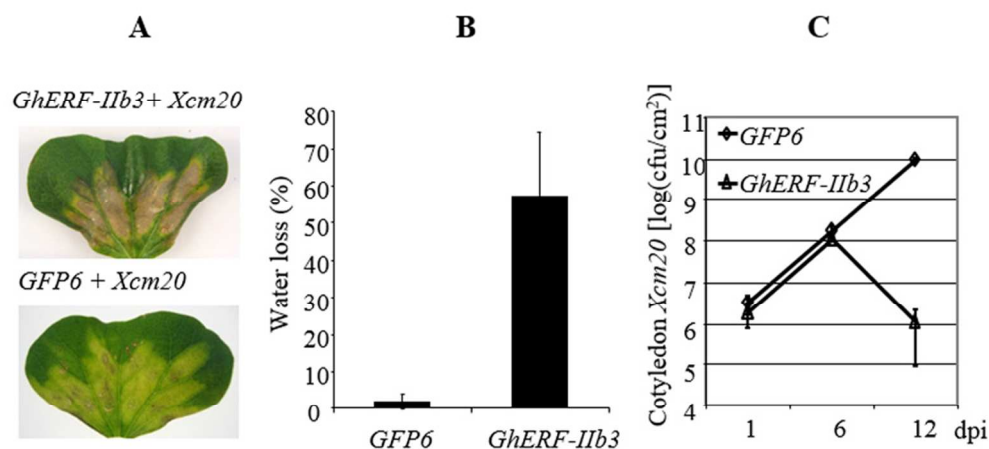


Figure 6. Overexpression of *GhERF-IIb3* induces bacterial blight resistance in cotton plants.

(A) Cotton cotyledons overexpressing *GhERF-IIb3* or *GFP6* as control were inoculated at 10^8 CFU/mL with the virulent *Xcm* strain (race20). Symptom photographs at 12 days post-inoculation (dpi). Note the HR-like symptoms in *GhERFIIb3*-expressing cotyledons compared to typical yellow disease symptoms in *GFP6* controls.

(B) Cell death intensity at 12 dpi using water loss as proxy.

(C) Kinetic evolution of cotyledon bacterial content.

All experiments (B, C and D) were repeated at least three times with similar results. Averages \pm SD (n=12) were calculated from biological triplicates.

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