RESEARCH CATEGORY: Plants Interacting with Other Organisms

Defense activated by 9-lipoxygenase-derived oxylipins requires specific mitochondrial proteins

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Key words: oxylipins/plant defense/mitochondria/cell wall signaling Arabidopsis

Running Titled: 9-Lipoxygenase defense signaling

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Abstract

9-Lipoxygenases (9-LOXs) initiate fatty acid oxygenation resulting in the formation of oxylipins activating plant defense against hemibiotrophic pathogenic bacteria. Previous studies using "noxy", a series of Arabidopsis mutants insensitive to the 9-LOX product 9-hydroxy-10,12,15-octadecatrienoic acid (9-HOT), have demonstrated the importance of cell wall modifications as a component of 9-LOX-induced defense. Here, we show that a majority (71%) of 41 studied noxy mutants have an added insensitivity to isoxaben, a herbicide inhibiting cellulose synthesis and altering the cell wall. The specific mutants noxy2, noxy15, and noxy38, insensitive to both 9-HOT and isoxaben, displayed enhanced susceptibility to Pseudomonas syringae DC3000 as well as reduced activation of salicylic acid-responding genes. Map-based cloning identified the mutation in noxy2 as At5g11630 encoding an uncharacterized mitochondrial protein, designated NOXY2. Moreover, noxy15 and noxy38 were mapped at the DRP3A (DYNAMIN RELATED PROTEIN) and FMT (FRIENDLY MITOCHONDRIA) loci, respectively. Fluorescence microscopic and molecular analyses revealed that the three noxy mutants characterized exhibits mitochondrial dysfunction, and that 9-HOT added to wild-type Arabidopsis causes mitochondrial aggregation and loss of mitochondrial membrane potential. The results suggest that the defensive responses and cell wall modifications caused by 9-HOT are under mitochondrial retrograde control, and that mitochondria play a fundamental role in innate immunity signaling.
Introduction

Plants have evolved distinct mechanisms to sense the presence of potential pathogens and respond by activating innate immunity. Compounds recognized by specific plant receptors include non-self structures derived from attacking pathogens such as conserved microbial molecules (designated as microbe-associated molecular patterns or MAMPs) and specific effector molecules delivered into the plant cell (Boller and Felix, 2009). Additionally, plants recognize self-structure signals released from pathogen-damaged tissues or induced during infection to potentiate defense and limit pathogen damage (designated as damage-associated molecular patterns or DAMPs) (Brutus et al., 2010; Yamaguchi et al., 2010). The plant responses to distinct recognized molecules share considerable overlap and converge in transcriptome reprogramming and generation of defense compounds (Dodds and Rathjen, 2010). Critical signals orchestrating the plant responses to pathogen attack are defense hormones such as salicylic acid (SA), jasmonates (JA) and ethylene (ET) (Glazebrook, 2005) and their cross talk with other signaling pathways (López et al., 2008; Robert-Seilaniantz et al., 2011). Moreover, other signaling molecules, including reactive oxygen species and nitric oxide, participate in triggering and regulating plant immunity (Torres et al., 2006; Vellosillo et al., 2010; Spoel and Loake, 2011). The interplay between distinct signaling molecules and their respective down-stream networks is complex and not fully understood.

The defense hormone JA belongs to a large family of active lipid derivatives, collectively known as oxylipins, whose importance in controlling pathological processes in plants is gradually being recognized (López et al., 2008; Andreou et al., 2009). Oxylipins are produced by initial oxidation of fatty acids, mainly linolenic (18:3) and linoleic (18:2) acids, by the action of lipoxygenases (9-LOX and 13-LOX), α-dioxygenases (α-DOX) or monoxygenases followed by secondary modifications catalyzed mainly by cytochrome P-450 enzymes or peroxygenase (Blée, 2002; Hamberg et al., 2002; Andreou et al., 2009). In addition, production of oxylipins from polyunsaturated fatty acids can take place non-enzymatically in the presence of singlet oxygen or by free radical-mediated oxygenation (Durand et al., 2009).

The jasmonate pathway of the oxylipin metabolome is initiated by 13-lipoxygenase (13-LOX) and results in the formation of (+)-7-iso-jasmonic acid (JA) and its isoelucine conjugate, compounds which control the resistance of plants to necrotrophic pathogens and insect attack (for recent reviews see Browse, 2009; Fonseca et al., 2009; Wu and Baldwin, 2010). In addition, other oxylipins have been found to exert defensive activities by acting as regulators of gene expression (Stintzi et
al., 2001; Browse, 2009; Mueller and Berger, 2009), cell death (De León et al., 2002; Hamberg et al., 2003; Montillet et al., 2005) and as sources of antimicrobial compounds (Prost et al., 2005). Recently, the importance of the 9-LOX pathway in the response of plants to pathogens and pests attack has been unraveled (Hwang and Hwang, 2010; Lopez et al., 2011; Nalam et al., 2012). Genetic studies in Arabidopsis thaliana revealed the role of 9-LOX oxylipins in activating local defense against virulent Pseudomonas (Hwang and Hwang, 2010; Lopez et al., 2011; Vicente et al., 2012) as well as the cooperation of the 9-LOX and α-DOX pathways in triggering systemic resistance (Vicente et al., 2012). Moreover, strong 9-LOX activity has been found in roots of Arabidopsis plants where 9-LOX products modulate root architecture (Vellosillo et al., 2007).

Studies of the signaling processes mediating the action of 9-LOX derivatives have indicated that their defense activities are in part exerted by modulating hormonal homeostasis (Lopéz et al., 2011; Vicente et al., 2012) as well as by inducing defense responses through a JA-independent signaling pathway that might be functionally involved in cell wall modifications (Vellosillo et al., 2007). To further investigate 9-LOX signaling, here we made use of noxy mutants (non-responding to oxylipins) having an impaired response to the 9-LOX derivative 9-HOT. We found that noxy mutants showing enhanced susceptibility to Pseudomonas syringae pv tomato (Pst) were partially insensitive to isoxaben, a herbicide inhibiting cellulose synthesis and triggering cell wall repair responses. Furthermore, identification and characterization of noxy mutations provided genetic evidence probing the contribution of mitochondria in signaling 9-LOX- and SA-upregulated defense.

Results

The noxy2 mutant, non-responding to 9-HOT, shows partial insensitivity to isoxaben.

In order to identify signaling components of the 9-HOT-induced defensive response we made use of noxy2-1 (non responding to oxylipins 2), a 9-HOT insensitive mutant that was isolated in a forward genetic screen based on the root waving activity of 9-HOT. This mutant displayed enhanced susceptibility to avirulent and virulent strains of Pseudomonas, reduced activation of 9-HOT-responses (root waving, formation of callose deposits and expression of 9-HOT-responding genes) and altered root development (Vellosillo et al., 2007). Genetic and map-based cloning studies indicated that the noxy2-1 mutation was monogenic and recessive, and was located in
At5g11630 encoding an uncharacterized protein that we named NOXY2. Confirmation of this was provided by the complementation of the noxy2-1 phenotypic alterations (root development and 9-HOT sensitivity) by transformation with a genomic clone of At5g11630 (Supplemental Fig. S1). Further data validating the identification of the noxy2-1 mutation was the observation that a T-DNA insertion mutant (SALK_019745) in At5g11630 (that we designated as noxy2-2) showed, like noxy2-1, 9-HOT-insensitivity and altered root development, and that both phenotypic alterations were restored by expression of a wild-type version of At5g11630 under the control of the 35S promoter (noxy2-2;35S:At5g11630) (Supplemental Fig. S1).

Studies on the response of plants to 9-HOT revealed that the defensive activity of this oxylipin was, at least in part, due to modification of the cell wall (Vellosillo et al., 2007). Therefore, we examined whether noxy2 mutants differed from wild-type plants in their response to isoxaben, a damaging herbicide inhibiting cellulose synthesis and triggering repair processes and up-regulation of defense-related genes (Caño-Delgado et al., 2003; Manfield et al., 2004; Bischoff et al., 2009; Denness et al., 2011). To this end, seeds from noxy2-1, noxy2-2 and wild-type plants were germinated in isoxaben-containing medium and examined seven days after germination. Two mutants, resistant (ixr1-1) or hypersensitive (prc1-1) to isoxaben, respectively, were included in these analyses (Fagard et al., 2000; Scheible et al., 2001). As shown in Figure 1 (panels A and B), isoxaben caused growth arrest, swelling and ectopic production of lignin in roots of wild-type seedlings. As expected, no such alterations were observed in ixr1-1 plants, in which mutation of the cellulose synthase CESA3 renders an active isoform insensitive to isoxaben (Scheible et al., 2001). Importantly, the two noxy2 mutants showed partial insensitivity to isoxaben. Thus, whereas isoxaben reduced the length of the primary root of wild-type plants by 83%, this effect diminished to 38% and 3%, in noxy2-1 and noxy2-2, respectively (Fig. 1C). In addition, microscopic examination revealed that formation of lignin deposits and root swelling in wild-type plants in response to isoxaben was much less pronounced in noxy2-1 and noxy2-2 mutants (Fig. 1, A and B). In contrast to the results with ixr1-1 and noxy2 mutants, a clear isoxaben hypersensitivity was found in prc1-1 plants, in which loss of CESA6 function caused a cellulose synthase defect and cellulose deficiency (Fagard et al., 2000) (Fig. 1, A, B and C).

**NOXY2 encodes a mitochondrial protein of unknown function**

Analyses of NOXY2 expression by means of promoter β-glucuronidase constructs (NOXY2:GUS) revealed GUS activity in cotyledons and roots of transgenic seedlings
and in floral tissues of mature plants (Fig. 2A). GUS staining was primarily localized in vascular tissues, lateral root primordia (LRP) and root meristems. Increased GUS staining was apparent in cotyledons and roots of 9-HOT- or isoxaben-treated seedlings (Fig. 2, A, B and D). Also, the expression of the NOXY2:GUS was induced in leaves inoculated with the avirulent bacterial strain Pst DC3000 avrRpm1 or the virulent bacterium Pst DC3000, as well as in response to isoxaben infiltration, whereas no induction was observed in water-infiltrated leaves used as a control (Fig. 2, C and D).

Examination of NOXY2 expression by using semiquantitative RT-PCR revealed the presence of two mRNAs, designated NOXY2-α and NOXY2-β, that accumulated in response to 9-HOT, bacterial inoculation and isoxaben (Fig. 2E). Cloning and sequencing of the corresponding cDNAs revealed that these mRNAs are splice variants of NOXY2 directing the synthesis of proteins (NOXY2-α and NOXY2-β) with 93 and 91 amino acids, respectively. The two proteins were identical in their first 82 amino acids but differed in their C-terminal regions (Fig. 2F). The NOXY2-α mRNA reached higher levels than NOXY2-β transcripts in the three responses examined, among which isoxaben caused the stronger induction. The phenotypic alterations and the responses to 9-HOT and isoxaben of noxy2-1 and noxy2-2 were restored by expression of a wild-type NOXY2-α cDNA under the control of the 35S promoter (35S:NOXY2-α) but not by expression of 35S:NOXY2-β, thus indicating a major role of NOXY2-α (Supplemental Fig. S2). In these lines, the expression of the two transgenes (35S:NOXY2-α and 35S:NOXY2-β) reached similar levels of accumulation (Supplemental Fig. S2), therefore indicating that the failure of NOXY2-β to complement the noxy2 phenotype was not due to low levels of expression, but that NOXY2-α is the functional NOXY2 protein.

Genome analyses indicated that Arabidopsis contains 11 NOXY2 homologs sharing 50 to 60% sequence identity with NOXY2. Nine of the NOXY2 homologs identified generate distinct mRNA variants encoding proteins that vary in their last amino acids but that are otherwise identical to each other (Supplemental Fig. S3). Apart from At2g20585 playing an unknown function in polar nuclei fusion during female gametophyte development (Portereiko et al., 2006), no other functions have been assigned to any NOXY2 homolog. Protein alignment revealed a highly conserved 20-amino acid region that contains the noxy2-1 mutation (a C to T transition at nucleotide 188 that converts Ala-63 to Val) and might thus be relevant for protein functionality (Supplemental Fig. S3).

Sequence analyses of NOXY2 indicated the presence of a signal peptide that might target mitochondrial localization. Therefore, we examined the subcellular location of NOXY2 by generating translational fusions in which the GFP protein was fused in
frame to the C-terminal of the NOXY2α cDNA. We used the 35S promoter to express the NOXY2α-GFP fusion (35S:NOXY2α-GFP) in wild-type controls and noxy2-1 mutants. The phenotype of noxy2-1 mutants was partially rescued by expression of the 35S:NOXY2α-GFP (Supplemental Fig. S4), and GFP fluorescence was primarily localized to small but distinct regions (Fig. 2G). Parallel staining with MitoTracker red, a dye that specifically accumulates in the mitochondria, revealed that GFP fluorescence (in 35S:NOXY2α-GFP transgenic plants) colocalized with the fluorescence of MitoTracker red indicating that NOXY2 is targeted to the mitochondria (Fig. 2G).

**9-HOT shares signaling events with those triggering cell wall repair responses**

Given that noxy2 was isolated together with additional noxy mutants in a screen for 9-HOT insensitive plants (Vellosillo et al., 2007), we predicted that additional noxy mutations should yield partial insensitivity to isoxaben. Therefore, we examined the phenotype of 40 noxy mutants grown in isoxaben-containing medium. In these analyses we found that 44% of noxy mutants respond like noxy2 plants with smaller reduction of root length, reduced swelling and reduced formation of lignin deposition compared with wild-type plants (see noxy15 and noxy38 as representative examples in Figure 3A), whereas 29% of noxy plants responded like wild-type plants to isoxaben (see noxy10 and noxy18 in Figure 3C). In addition, an intermediate response to isoxaben was found in 27% of noxy mutants that were deficient in lignin deposition and root swelling, but otherwise showed a similar reduction in root length as seen in wild-type plants (see noxy21 and noxy33 as representative examples in Figure 3B). The fact that 71% of noxy mutants were partially insensitive to isoxaben indicates that the noxy mutations might impair the ability of plants to activate cell wall repair responses and that the responses to 9-HOT share signaling events with those involved in maintaining the integrity of the cell wall. The finding that the cellulose deficient prc1-1 mutant, like the noxy plants was partially insensitive to 9-HOT supported the interplay between the two pathways. (Fig.1D).

**NOXY2, NOXY15 and NOXY38 contribute to plant defense against virulent bacterial pathogens**

Among the 9-HOT insensitive mutants characterized above, noxy15 and noxy38 respond like noxy2 to isoxaben and were selected for further examination. Since noxy2-1 showed enhanced susceptibility to Pseudomonas infection (Vellosillo et al.,
2007), it seemed likely that also the noxy15 and noxy38 mutations should diminish the defense potential of plants. Therefore, we examined the phenotype of noxy15 and noxy38 for enhanced susceptibility to Pst DC3000. The noxy2-2 mutant was also included in these studies. No major alterations of the plant phenotype (Supplemental Fig. S5), except for slower shoot growth compared to wild-type was observed in noxy mutants. Three days after bacterial inoculation, growth of Pst DC3000 in leaves of noxy2-2, noxy15 and noxy38 mutants was higher by factors of 5, 10, and 6, respectively, compared to wild-type controls (Fig. 4A). Accordingly, noxy2-2, noxy15 and noxy38 showed stronger disease symptoms than those seen in wild-type plants (Fig. 4B).

A major signal controlling the activation of plant defense against Pst DC3000 infection is salicylic acid (SA). Therefore, to further characterize the noxy mutations we examined SA-dependent gene expression after SA application. In line with the enhanced susceptibility of noxy mutants, transcript abundance of the two SA markers examined, PR1 and PR5, was lower in noxy mutants than in wild-type plants (Fig. 4, C and D). The diminished capacity of noxy mutants to activate SA-mediated up-regulation of gene expression might be at least partly explain the reduced defensive potential seen in noxy mutants. Moreover, the results indicated that the NOXY2, NOXY15 and NOXY38 proteins play a positive role in plant defense where their activity would contribute to the activation of SA-dependent defense gene expression.

The noxy2, noxy15 and noxy38 mutations disturb mitochondrial morphology and distribution

Map-based cloning studies indicated that the noxy15 mutation was located in At4g33650, encoding the dynamin-related protein DRP3A known to localize to the outer surface of mitochondria and to play a key role in mitochondrial fission (Fujimoto et al., 2009). The noxy15 mutation was a G to A transition at nucleotide 281 that converts Arg-94 to His at the putative GTPase domain of the DRP3A protein and was renamed drp3a-1 (Supplemental Fig. S6). Confirmation that noxy15 impairs mitochondrial fission was obtained by transformation with a mitochondrial-YPF marker (mt-CD3-990; Nelson et al., 2007) allowing mitochondrial visualization. Thus, whereas small and discrete mitochondria were visible in transgenic wild-type plants (Col-0;35S:Mt-YFP) (Fig. 5A), mitochondria in transformed noxy15 plants (noxy15;35S:Mt-YFP) appear connected to each other forming a network of massively elongated mitochondria and large nodule-like structures that likely correspond to mitochondrial aggregates (Fig. 5B).
The noxy38 mutation was mapped at the FMT locus (FRIENDLY
MITOCHONDRIA, At3g52140), a homolog of the CluA gene of Dicyostelium discoideum encoding a predicted TPR protein (tetratricopeptide repeat) playing a role in the correct distribution of mitochondria in the cell (Logan et al., 2003). The noxy38 mutation is a G to A transition in the last nucleotide of intron 22 and was renamed fmt-1 (Supplemental Fig. S6). Transcripts derived from fmt-1 include intron 22 and are translated into a predicted truncated protein of 1053 amino acids lacking the TPR domain, thought to function in protein-protein interactions. The subcellular localization of FMT is presently unknown, however, in line with previous reports (Logan et al., 2003), Mitotracker staining of wild-type and noxy38 roots reveals the formation of strong mitochondrial aggregates in noxy38 plants (Fig. 5, C and D).

Because mitochondrial morphology and distribution was altered in noxy15 and noxy38 mutants we examined these organelles in noxy2 mutants after transformation with the mitochondrial-YFP marker (mt-CD3-990; Nelson et al., 2007). Although most mitochondria showed a wild-type distribution and size, nodule-like structures were visible in noxy2-1 (noxy2-1;35S:Mt-YFP) and noxy2-2 transgenic plants (noxy2-2;35S:Mt-YFP) (Fig. 5, E and F).

The noxy2, noxy15 and noxy38 mutations alter mitochondrial functionality

Given that plants integrate mitochondrial signals to regulate nuclear gene expression, referred to as retrograde signaling (Rhoads et al., 2006), we predicted that the mitochondrial alterations in noxy mutants could negatively impact mitochondrial-nuclei communication. To examine this possibility, we analyzed the expression of mitochondrial alternative oxidase 1 (AOX1a; At3g22370) shown to be under retrograde regulation in response to specific mitochondrial damaging agents such as antimicyn A (Rhoads et al., 2006). Additionally, the expression of a mitochondrial manganese superoxide dismutase (MSD1; At3g10920) controlling ROS production (Morgan et al., 2008) was similarly examined. Analyses of AOX1a and MSD1 transcript abundance after antimicyn A revealed significant differences between wild-type plants and noxy mutants (Fig. 5, G and H). The level of AOX1a transcripts reached higher levels in noxy15 and noxy38 mutants than in wild-type plants, whereas the opposite was observed in noxy2-2. In the case of MSD1 we observed a weak induction above basal levels in wild-type plants, whereas the level of transcripts in the noxy plants examined decreased as compared with wild-type plants.

The results of these analyses indicated that the three noxy mutations characterized caused an alteration in the response of plants to mitochondrial damage.
and thus, that the noxy mutations might disturb mitochondrial functionality.

**Effect of 9-HOT on mitochondria**

Given that noxy mutants disturb mitochondrial morphology and function we examined whether the application of 9-HOT might exert any effect on mitochondria. As seen in Figure 6A, treatment of seedlings with 9-HOT provoked mitochondrial swelling and aggregation as well as the formation of mitochondrial structures of annular appearance. Clusters of round-shaped mitochondria were preferentially seen in the differentiation zone of the roots, whereas annular structures were mainly found in hypocotyls. Because mitochondrial swelling is frequently associated with a loss of membrane potential (Borutaite, 2010; Schwarzländer et al., 2012) we also tested whether the application of 9-HOT decreases the membrane potential of mitochondria. In order to assess this possibility, Col-0 plants expressing the mt-YFP fluorescence marker (Nelson et al., 2007) were stained with tetramethyl rhodamine methyl ester (TMRM), a dynamic reporter of mitochondrial membrane potential whose fluorescence is spectrally separated from that of mt-YPF. As expected, a red signal colocalizing with the mitochondrial-YPF protein was seen after TMRM staining (Fig. 6B). The red and green fluorescence intensities from individual mitochondria were similar in control untreated samples, however, an overall decrease of the TMRM fluorescence (~33%) was seen after the application of 9-HOT (Fig. 6C). The reduction of red fluorescence indicated that 9-HOT caused loss of mitochondrial membrane potential and membrane depolarization.

**Discussion**

Previously we showed that the 9-LOX oxylipin pathway participates in the defense of *Arabidopsis* against hemibiotrophic bacteria, and found evidence that the 9-LOX-derivative 9-HOT induces cell wall modifications restricting pathogen invasion. Here, we made use of 9-HOT-insensitive mutants (*non-responding to oxylipins*) to further examine the molecular events involved in 9-LOX signaling. The noxy2 mutant showing enhanced susceptibility to avirulent and virulent strains of *Pseudomonas* was first selected for this study.

In accordance with the role of 9-LOX in plant defense (Hwang and Hwang, 2010; López et al., 2011; Vicente et al., 2012) we found that the expression of NOXY2 was induced by 9-HOT and bacterial infection. Moreover, consistent with the induction of cell wall changes by 9-HOT, we found that expression of NOXY2 was induced by
isoxaben, an herbicide inhibiting cellulose biosynthesis and triggering cell wall repair and defense responses (Caño-Delgado et al., 2003; Manfield et al., 2004; Bischoff et al., 2009; Denness et al., 2011). The finding that the 9-HOT-insensitive \textit{noxy2} was also partially insensitive to isoxaben indicated that the response of plants to these two compounds share common signaling processes, a conclusion that was strongly supported by the results showing that out of the 41 \textit{noxy} mutants tested (isolated together with \textit{noxy2} in a screen for 9-HOT-insensitive plants), 71% showed partial insensitivity to isoxaben. These results are consistent with a failure of \textit{noxy} mutants in signaling cell wall repair responses and suggested the participation of a high proportion of NOXY proteins in this regulatory pathway. Moreover, the fact that the cellulose-deficient \textit{pcr1-1} mutant was insensitive to 9-HOT (Fig. 1D) suggested that \textit{noxy} mutants might be also altered in the composition of the cell wall. Further examination will be required to ascertain this possibility.

Control of cell wall integrity in plants is needed to allow growth and cell expansion under physiological conditions (Wolf et al., 2012). Moreover, repair and cell wall strengthening mechanisms are critical defense responses to limit pathogen infection (Boller and Felix, 2009; Brutus et al., 2010; Yamaguchi et al., 2010; Denness et al., 2011). In this context, the fact that the 9-HOT insensitive \textit{noxy2-2}, \textit{noxy15} and \textit{noxy38} mutants displayed enhanced susceptibility to \textit{Pseudomonas} infection suggested the participation of NOXY proteins in the signaling processes mediating cell wall-mediated defense. In addition, these results support previous findings pointing to the involvement of 9-HOT and of the 9-LOX oxylipin pathway in activating cell wall-defense responses (Vellosillo et al., 2007). Previous studies with \textit{noxy} mutants demonstrated that enhanced bacterial susceptibility and 9-HOT insensitivity can be the consequence of a constitutive production of ethylene (ET) (López et al., 2011). However, we note that the phenotype of \textit{noxy2-2}, \textit{noxy15} and \textit{noxy38} does not show the alterations associated with the activation of the ET pathway (short hairy roots) and thus, it is unlikely that the defects produced by these mutations could be due to the overproduction of ET.

The identification of NOXY2 as a functionally unknown mitochondrial protein and of \textit{noxy15 (drpa3-1)} and \textit{noxy38 (fmt-1)} as new alleles of \textit{DRP3A} encoding a mitochondrial dymanin-related protein (DRPA3) (Fujimoto et al., 2009) and \textit{FMT} (FRIENDLY MITOCHONDRIA) serving for the distribution of mitochondria in the cell (Logan et al., 2003), respectively, indicated the participation of mitochondria in signaling the response to 9-HOT and in plant defense. Of interest, information found in databases and our own experiments indicated that the three genes identified (\textit{NOXY2}, \textit{NOXY15/DRP3A} and \textit{NOXY38/FMT}) generate distinct mRNA transcripts and thus, that the regulation of their expression is subjected to alternative splicing, a widespread
mechanism to regulate both protein function and gene expression in plants (Filichkin et al., 2010). Detailed studies on NOXY2 gene expression revealed that the two spliced variants identified were induced in response to 9-HOT, bacterial inoculation and isoxaben and that the relative accumulation of the two transcripts examined did not vary during the treatments analyzed (Fig. 2). Besides, we found that only one of the two predicted NOXY2 encoded proteins, namely NOXY2-α, complemented the phenotypic alterations found in noxy2 mutants and thus, that this protein variant corresponded to the active NOXY2 protein.

Visualization of mitochondria in noxy mutants revealed clear alterations in the shape and distribution of these organelles as compared with wild-type plants. This was also the case after examining the response of plants to specific mitochondrial damaging agents such as antimycin A, which can result in the production of mitochondrial ROS (Chang et al., 2012). Because alternative oxidase functions to alleviate mitochondrial stress (Rhoads et al., 2006), the enhanced induction of AOX1α expression in noxy15 and noxy38 mutants is consistent with an enhanced production of stress signals. By contrast, the reduction of AOX1α expression in noxy2-2 could result from a failure in sensing or responding to mitochondrial damage. Regardless of the detailed mechanisms involved, it may be concluded that the alteration of gene expression observed in the noxy mutants examined is likely reflecting a functional perturbation of the mitochondria. Further support for this conclusion came from the results showing reduced expression in noxy mutants of MSD1 encoding a mitochondrial manganese superoxide dismutase that controls ROS production (Morgan et al., 2008).

Additional evidence supporting the participation of mitochondria in 9-HOT signaling was the observation that 9-HOT provoked the formation of mitochondrial aggregates and annular mitochondrial structures as well as a loss of membrane potential and as a consequence, the depolarization of the mitochondrial membrane. Mitochondrial changes similar to those induced by 9-HOT were produced after generation of superoxide ion in Arabidopsis plants (Scott and Logan 2008). Moreover, mitochondrial swelling and aggregation was found to accompany the response to elevated temperatures in yeast (Kawai et al., 2001) and to precede apoptosis and innate immune responses in vertebrates where aggregation of these organelles might help to increase the concentration of signals (Haga et al., 2003; Arnoult et al., 2011). Similarly, the loss of membrane potential has been shown to be associated with mitochondrial swelling, which in turn might serve to facilitate solute exchange and the communication between the mitochondria and the cytosol (Borutaite, 2010; Schwarzländer et al., 2012).
Numerous studies have proven the direct link between mitochondria and innate immunity signaling in vertebrates, in which these organelles play a crucial role in regulating host immunity (Arnoult et al., 2011). Also, a role of these organelles in plant defense responses is emerging from recent results showing that, as found in noxy mutants, suppression of innate immunity and reduced SA-upregulation of gene expression may be linked to mitochondrial dysfunction (Block et al., 2010; Gleason et al., 2011), and that up-regulation of defense gene expression might be under mitochondrial retrograde control (Schwarzländer et al., 2012). The results point to the participation of mitochondria during the activation of plant immunity resulting in a robust resistance response to protect plant tissues against pathogen infection.

Materials and Methods

Plants and Growth Conditions

Arabidopsis thaliana wild type plants and ixr1-1 and prc1-1 mutants, used in this study were derived from Arabidopsis plants ecotype Columbia (Col-0). For in vitro analyses, sterilized seeds were vernalized for 3 days at 4°C, and allowed to grown on media plates. Growth conditions were 14 hours of light, 10 hours of dark, 22°C and 250 μE m⁻² sec⁻¹ fluorescent illumination. For phenotypic examination after 9-HOT and isoxaben treatments, seeds were germinated in vertical plates and transferred 4-days after germination to plates containing the products of interest (9-HOT: 25 μM and isoxaben: 10 nM). For examining gene expression seeds were germinated in horizontal plates and treated 12-days after germination with SA (250 μM) and AA (20 μM). For in planta analyses seeds were sown on soil, vernalized for 3 days at 4°C, and grown in chamber at 22°C and 70% relative humidity under a 14 hours light, 10 hours dark photoperiod at 250 μE m⁻² sec⁻¹ fluorescent illumination. Plants were treated and examined between 3 and 4 weeks after seed germination.

In vivo Analyses of Bacterial Growth

Bacterial inoculations were performed under greenhouse conditions by spraying a suspension (10⁸ cfu/mL) onto the leaf surface. The bacterial strains used in this study were Pst DC3000 avrRpm1 (avirulent) and Pst DC3000 (virulent). Prior to spray inoculation, bacteria were grown overnight at 28°C in Petri plates with King's B medium. Bacterial suspensions were prepared in 10 mM MgCl₂ containing 0.04% Silwet L77. Discs from infected leaves were excised at the times of interest, pooled in triplicate, homogenized and used for counting bacterial growth in Petri plates. Reported
results are the means and standard errors of the values obtained in three independent experiments.

**Preparation of 9-HOT**

9(S)-Hydroxy-10(E),12(Z),15(Z)-octadecatrienoic acid (9-HOT) was prepared by stirring linolenic acid (120 mg) at 23°C with tomato whole homogenate under an atmosphere of oxygen essentially as described by Matthew et al. (1977). The product was subjected to open column silicic acid chromatography to provide >95% pure 9(S)-HPOT (70 mg). Treatment with sodium borohydride (100 mg) in methanol (10 mL) at 0°C for 30 min followed by preparative straight-phase HPLC using 2-propanol/hexane/acetic acid (2.2:97.8:0.005, v/v/v) as the mobile phase afforded >99% pure 9-HOT as a colorless oil (44 mg; yield, 35% from starting linolenic acid). 9-HOT stock was prepared in 95% ethanol and diluted with water to reach the concentration used in these studies.

**RNA Isolation and Analyses of Gene Expression**

For extraction of RNA, plant tissues were collected at different time intervals, frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA was isolated according to Logemann et al. (1987). Reverse transcription (RT)-PCR was performed with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems) by using the Titan One Tube RT-PCR System (Roche Applied Science, Mannheim, Germany) as specified by the manufacturer. Total RNA was treated with DNase TURBO DNA-free (Ambion, Austin, TX, USA) to remove contaminating DNA. 100 ng of this RNA were used in each one-step RT-PCR reaction. Amplification and sequencing of NOXY2 cDNAs was performed with 5’ and 3’ Rapid Amplification cDNA ends (5’ and 3’ RACE) (Invitrogen, Darmstadt, Germany). Quantification of gene expression was performed by qRT-PCR analyses using a GenAmp PCR system 9700 thermal cycler (Applied Biosystems) and the FastStart Universal SYBR Green Master (Rox) system as specified by the manufacturer (Roche). Gene At1g43170 encoding RPL3A was used as an internal control. Primers used for these analyses and lengths of amplification products are described in Supplemental Table S1.

**Construction of chimeric constructs and generation of transgenic lines**

Genomic and cDNA NOXY2 sequences were used to prepare chimera constructs using standard procedures. DNA fragments were PCR-amplified using Expand High Fidelity polymerase (Roche, Mannheim, Germany). Forward and reversed primers used in each case are shown in Supplemental Table S1. DNA amplified PCR-
fragments were inserted into appropriated GATEWAY plasmids and mobilized to A. tumefaciens for plant transformation.

**Mapping and cloning of noxy2-1, noxy 15 and noxy38 genes**

For mapping purposes, noxy2-1, noxy15 and noxy38 plants (derived from the Col-0 accession) were crossed to wild-type plants of the Landsberg erecta ecotype (Ler) and F2 mutants were selected. DNA from noxy2-1 and noxy15 recombinants was prepared and used to analyze linkage of the mutations to SSLP and CAPS markers (http://www.arabidopsis.org, http://bar.utoronto.ca). Sequencing of candidate genes was performed to identify the noxy2-1 and noxy15 mutations. Mapping of the noxy38 was performed at the IB-UMH Gene Mapping Facility (Elche Spain) as described by Ponce et al. (2006). In brief, for low-resolution mapping, the DNA of 50 F2 phenotypically mutant plants was individually extracted and used as a template to multiplex PCR coamplify 32 SSLP and In/Del molecular markers using fluorescently labelled oligonucleotides as primers. For fine mapping, 400 additional F2 plants were used to assess linkage between noxy38 and molecular markers designed according to the polymorphisms between Ler and Col-0 described at the Monsanto Arabidopsis Polymorphism Collection database (http://www.arabidopsis.org). Massive genome sequencing performed at BIG genomic (http://www.genomics.cn) was used for the identification of the noxy38 mutation.

**Protein sequence analysis**

NOXY2 homolog proteins were identified in databases using the BLAST program available at NCBI GenBank and TAIR. Multiple sequence alignment was performed using ClustalW sequence program. Alignment was graphically displayed using the Jalview alignment editor with Clustalx colour scheme (Waterhouse et al., 2009).

**Histochemical analyses**

Examination of GUS activity in NOXY2:GUS transgenic plants was performed as described in Vellosillo et al. (2007). For the detection of callose, plant tissues were stained and visualized as described in Vellosillo et al. (2007). For the detection of lignin, roots were stained with phrologlucinol-HCl 2% as described in Schrick et al. (2004). For visualization of mitochondria roots were stained with MitoTracker Red CMXRos (M-7512, Invitrogen) as described in Hedtke et al. (1999). The membrane potential of mitochondria was assessed in hypocotyl cell by using the cationic lipophilic fluorescent dye tetramethyl rhodamine methyl ester (TMRM). Whole seedlings were equilibrated in 25 nM TMRM (in 0.5 X MS medium) for 30 min before use. For
treatment with 9-HOT seedlings were incubated in 0.5X MS medium containing 25 μM 9-HOT for 30 min. Fluorescent images of GFP, MitoTracker and TMRM were generated on a Leica TCS-SP5 confocal microscope with LAS AF v. 2.6.0 software, using a 63x/1.2NA water immersion objective and sequential scanning with Argon 488 nm (GFP) and DPSS 561 nm (MitoTracker and TMRM) laser lines.

ACKNOWLEDGMENTS

We are grateful to Shauna Somerville, Chris Somerville, Roberto Solano and Yovanny Izquierdo for critical reading of the manuscript and stimulating discussions; Marta Martínez for help with expression analyses; Juan Carlos Sánchez Ferrero for protein sequence analyses; R. Piqueras for help with in vitro plant growth; I. Poveda for expert photographing, and G. Hamberg (Karolinska Institutet, Stockholm, Sweden) for assistance during the preparation of the oxylipins used. The authors wish to thank María Rosa Ponce and Jose Luis Micol (Instituto de Bioenginería, Universidad Miguel Hernández, Elche, Spain) for their help with the positional cloning of the NOXY38 gene. The T-DNA insertion line used in this study was from the SALK collection and obtained from the NASC Arabidopsis Stock Center (http://arabidopsis.info). This work was supported by grants BIO2009-09670 and CSD2007-00057. Michael Bartsch was supported by a Marie Curie Fellowship of the EU (grant agreement Nº PIEF-GA-2008-221448).

REFERENCES


Fujimoto M, Arimura S, Mano S, Kondo M, Saito C, Ueda T, Nakazono M, Nakano A, Nishimura M, Tsutsumi N (2009) Arabidopsis dynamin-related proteins DRP3A and DRP3B are functionally redundant in mitochondrial fission, but have distinct roles in peroxisomal fission. Plant J 58: 388-400


FIGURE LEGENDS

Figure 1. Root phenotypes of wild-type, noxy2-1, noxy2-2, ixr1-1 and prc1-1 plants responding to isoxaben.

A, Transmitted light visualization of seedling grown for 7 d in control MS medium and in isoxaben-containing medium (10 nM).

B, Examination of ectopic lignin in roots of isoxaben-treated seedlings stained with phloroglucinol.

C, Length of primary roots in 7-d-old seedlings grown in control MS medium and in isoxaben-containing medium (10 nM). The data presented are results obtained in three independent experiments. Means and standard errors of 60 seedlings are shown.

D, Phenotype of seedlings grown for 3 d in MS medium and then transferred to grow for 3 additional days on 9-HOT-containing medium (25 μM) (upper panels). Fluorescence visualization of callose deposition in 9-HOT-treated roots stained with aniline blue (lower panels).

Scale bars = 50 μm.

Figure 2. Analysis of NOXY2 gene expression and localization of NOXY2 protein.

A, GUS activity in seedlings, roots, cotyledons and flowers of transgenic lines containing a NOXY2 promoter GUS construct (NOXY2:GUS).
B, GUS staining of transgenic seedlings grown in presence of 9-HOT (25 μM).
C, Visualization of GUS activity in mature leaves of NOXY2::GUS plants inoculated with water (mock), Pst DC3000 avrRpm1 and Pst DC3000 (10⁸ cfu/mL).
D, GUS staining in seedlings of transgenic plants grown in presence isoxaben (10 nM) (left panels) and leaves infiltrated with water (mock) or isoxaben (100 nM) (right panels).
Scale bars = 50 μm.
E, RT-PCR analysis of NOXY2 transcripts in leaves of wild-type plants responding to Pst DC3000 avrRpm1 and Pst DC3000 (10⁸ cfu/mL) inoculation and in roots of seedlings responding to 9-HOT (25 μM) and isoxaben (100 nM). Two DNA fragments corresponding to distinct spliced variants of NOXY2 were identified and designated as α and β, respectively.
F, Representation of the NOXY2 gene with exons shown as blue boxes. Arrowheads indicated splicing sites (numbered from I to V) and lines above and below the NOXY2 gene show the splicing events generating transcripts β and α, respectively. Alignment of NOXY2-α and NOXY2-β encoded proteins. C-terminal regions differing between NOXY2-α and NOXY2-β are shown in red.
G, Subcellular localization of NOXY2 in transgenic noxy2-1 mutants expressing a NOXY2α-GFP fusion (green). Roots from 7-d-old seedlings expressing the indicated fusion protein were staining with MitoTracker Red CMXRos staining (red). The GFP and MitoTracker images were merged to show the colocalization of the GFP signal at the mitochondria. Scale bars = 25 μm.

**Figure. 3.** Phenotypes of noxy mutants responding to isoxaben.
A, Transmitted light visualization of noxy15 and noxy38 seedlings grown for 7 d in control MS medium and in isoxaben-containing medium (10nM).
B, Phenotype of noxy21 and noxy33 seedlings grown as in panel A.
C, Phenotype of noxy10 and noxy18 seedlings grown as in panel A.
Histological examination of ectopic lignin accumulation in isoxaben-treated roots stained with phloroglucinol is shown. Scale bars = 50 μm.

**Figure. 4.** Response of wild-type, noxy2-2, noxy15 and noxy38 plants to bacterial infection.
A, Bacterial growth in wild-type, noxy2-2, noxy15 and noxy38 plants at 72 h after spray inoculation of a suspension of Pst DC3000 (10⁸ cfu/mL). Means and standard errors obtained in three independents experiments are shown. Letters on top of the bars
indicate statistically significant differences between wild-type and noxy plants (t-test; P < 0.001 (a), 0.001 < P < 0.01 (b) and 0.01 < P < 0.05 (c)).

B, Lesion developed in wild-type, noxy2-2, noxy15 and noxy38 plants. Shown are representative examples of symptoms developed at 72 h after spray inoculation.

C, Expression of PR1 was determined by qRT-PCR in RNA samples extracted at intervals times from SA (250 μM) treated seedlings. Gene At1g43170 encoding RPL3A was used to normalize transcript levels in each sample. Results shown are the mean of three independent experiments.

D, Expression of PR5 was determined as described in panel C.

**Figure. 5.** Mitochondrial visualization in roots of Col-0, noxy2-2, noxy15 and noxy38 plants.

A, Images of mitochondria (green) in transgenic roots of Col-0 expressing a 35S:Mt-YFP construct.

B, Images of mitochondria (green) in transgenic roots of noxy15/drp3a-1 plants expressing a 35S:Mt-YFP construct.

C, Mitochondrial (red) visualization in roots of Col-0 stained with MitoTracker Red CMXRos.

D, Mitochondrial (red) visualization in roots of noxy38/fmt-1 plants stained with MitoTracker Red CMXRos.

E, Images of mitochondria (green) in roots of noxy2-1 mutant expressing a 35S:Mt-YFP construct.

F, Images of mitochondria (green) in roots of noxy2-2 mutant expressing a 35S:Mt-YFP construct.

Scale bars = 10 μm.

G, Expression of AOX1a was determined by qRT-PCR in RNA samples extracted at different intervals from antimicyn A treated seedlings (20 μM). Gene At1g43170 encoding RPL3A was used to normalize transcript levels in each sample. Results shown are the mean of three independent experiments.

H, Expression of MSD1 was determined as described in panel G.

**Figure. 6.** Mitochondrial aggregation and decrease of membrane potential in response to 9-HOT.

A, Mitochondrial visualization of 9-HOT (25 μM) treated transgenic lines expressing 35S:Mt-YFP revealed the formation of aggregates (upper panels) and annular structures (lower panel).
B, TMRM mitochondrial staining (red) and green fluorescence of seedlings expressing 35S:mt-YFP (green). Merge of red and green coloration revealed similar fluorescence intensities in individual mitochondria of untreated samples (upper panels) and a reduction of TMRM fluorescence after the application of 9-HOT (25 μM) (lower panels). Scale bars = 10 μm.

C, Representation of the TMRM and mt-YFP fluorescence intensities measured in untreated and 9-HOT-treated roots. Means and standard errors of measurements of 15 images are shown. Letter on top of the bars indicate statistically significant differences between control and 9-HOT treatment (t-test; 0.001 < P < 0.01 (b)).