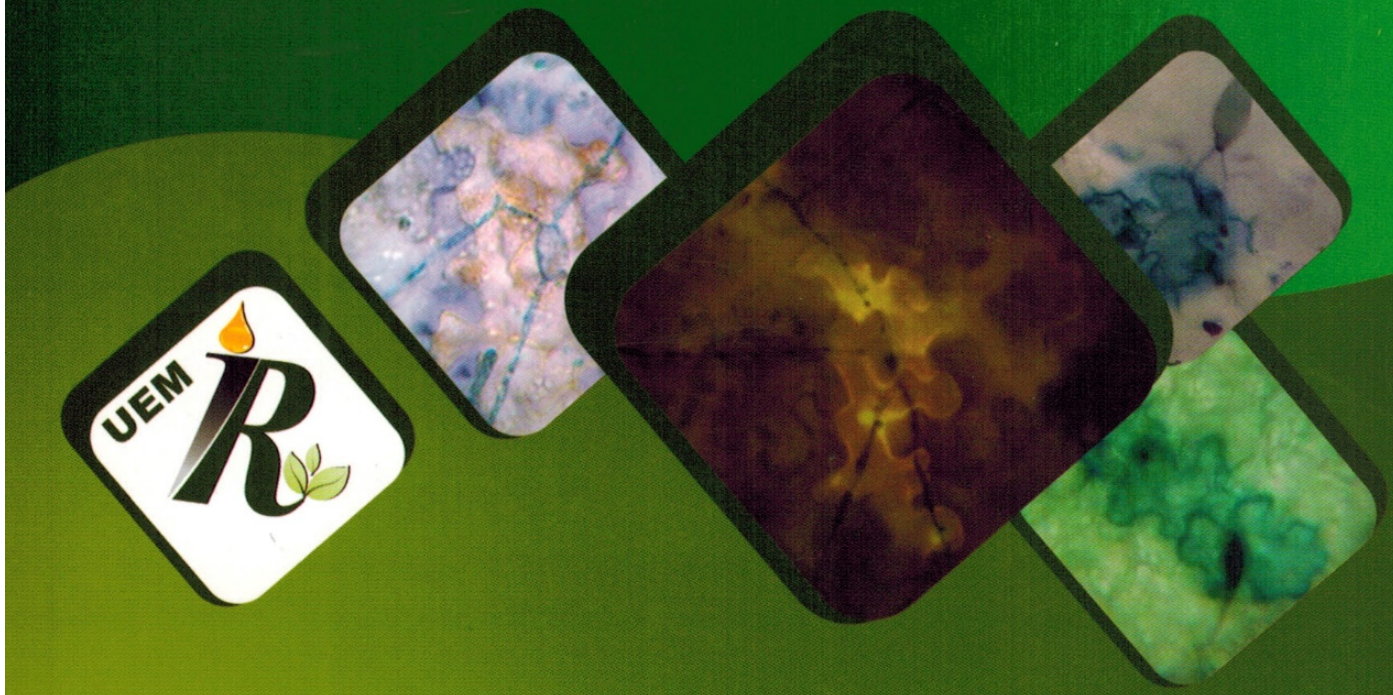

INDUÇÃO DE RESISTÊNCIA EM PLANTAS A PATÓGENOS

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Title: **Arachidonate-induced immunity and mixed messages in plant-oomycete interactions**

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Abstract: Plants rely on an array of phytohormones to coordinate responses to biotic and abiotic stresses. Defense response networks regulated in part by salicylic acid (SA) and jasmonic acid (JA) are impacted by the action of microbe-associated molecular patterns (MAMPs) and effectors secreted during infection. The eicosapolyenoic acids (EP) – arachidonic (AA) and eicosapentaenoic (EPA) acids – are common polyunsaturated fatty acids in oomycete pathogens that serve as novel MAMPs to induce resistance in plants. Plants do not produce EP. However, Arabidopsis plants engineered to express low levels of EP display altered tolerances to aphids and oomycete, fungal and bacterial pathogens compared to wild type plants. The presence of minor levels of AA in plant lipids alters JA- and SA-mediated gene expression and metabolite networks in a manner consistent with the disease phenotypes observed. The tomato-*Phytophthora capsici* interaction provides an important crop model to determine EP mode of action. Pre-treatment of tomato roots with EP induces resistance to *P. capsici* and primes the plant to respond with rapid lignification at infection sites while reducing crown rot and shoot collapse. Treatment with AA, but not linoleic acid (LA) or water as controls, strongly induces the expression of genes encoding 9-oxylipin pathway enzymes in tomato roots. Studies on oxylipin metabolism in plant immunity as well as recent work on plant activators that induce resistance in tomato in different stress contexts are presented.

Keywords: eicosapolyenoic acids, induced resistance, oxylipin, *Phytophthora capsici*, predisposition, *Solanum lycopersicum*

Introduction

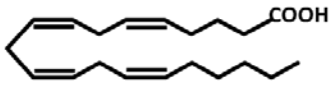
Plants rely on an array of phytohormones to coordinate responses to biotic and abiotic challenges. There is considerable crosstalk among the induced signals resulting in a complex and dynamic interplay that shapes the stress response, often allowing plants to adapt to or defend against the challenge. Interacting stresses can also weaken plants to predispose them to pathogens or levels of inoculum they would normally resist. How plants negotiate these diverse challenges is an important area of inquiry in plant biology. Delineation of these stress network interactions has implications for disease management, particularly in the deployment of chemically- or biologically-induced resistance. Studies with salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) have informed much of the current understanding of induced resistance in plants. However, other phytohormones induced by abiotic stresses, notably abscisic acid (ABA), can interfere with induced resistance.

Plants and animals recognize certain molecular signatures in microbial cells, triggering a form of immunity that may help the organism resist infection by potential pathogens. These molecular signatures, called microbe-associated molecular patterns (MAMPs)[1], are found in diverse molecules, including proteins, polysaccharides, and lipids (Boller and Felix, 2009). In plants, this pattern-triggered immunity (PTI) is characterized by a set of responses to delimit the pathogen which may include generation of reactive oxygen species (ROS), pathogenesis-related (PR)-proteins, programmed cell death, phytoalexin biosynthesis, callose and lignin deposition, and changes in levels and signaling of ET, SA, and JA (Tsuda et al., 2009). MAMPs that trigger plant defense responses also can trigger innate immune responses in animals, suggesting a degree of functional conservation across kingdoms of core signaling features (Zipfel and Robatzek, 2010). Studies of PTI have focused on the bacterial peptides flagellin and EF-Tu in Arabidopsis leaves. These peptides are perceived by plant pattern recognition receptors (PRRs), receptor-like kinases that are crucial for flagellin/EF-Tu action (Segonzac and Zipfel, 2011). Although many microbial products, historically referred to as elicitors, have been shown to elicit PTI in plants, most (unlike flagellin and EF-Tu) have not been investigated sufficiently to resolve their mode of action nor have they been investigated in organs such as roots (Millet et al., 2010).

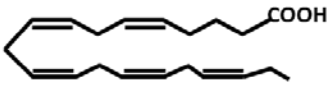
[1] Another term, PAMP, for pathogen-associated molecular pattern, is also used in the literature.

Arachidonate-triggered immunity

Eicosapolyenoic acids (EP) – arachidonic (AA) and eicosapentaenoic (EPA) acids – are common polyunsaturated fatty acids in lipids and other cellular components of plant pathogenic oomycetes that upon release during infection can serve as novel MAMPs to engage defense signaling networks (**Fig. 1**). These changes are manifested as a generalized rapid stress response resulting in enhanced tolerance to certain pathogens and insects (Bostock et al., 2011). Among plant pathogens, the capacity for EP synthesis appears to be largely restricted to oomycetes, a few primitive fungi (e.g., zygomycetes and chytrids) (Weete, 1974), and nematodes (Hutzell and Krusberg, 1982). Our research indicates that plant oxylipin metabolism plays a critical role in EP signal-response coupling to trigger immunity, but the exact mechanisms are unresolved (Bostock et al., 2011).



arachidonic acid (AA, 20:4 $\Delta^{5,8,11,14}$)



eicosapentaenoic acid (EPA, 20:5 $\Delta^{5,8,11,14,17}$)

Fig. 1. Structures of the eicosapolyenoic acids, arachidonic acid and eicosapentaenoic acid.

Previous research on EP indicates that their initial perception is likely different than other MAMPs, with the possibility that plant cells produce novel oxylipins from EP (Preisig and Kuć, 1988; Bostock et al., 1992). Oxylipins are secondary metabolites derived from lipids in animals and plants that are formed through enzymatic and non-enzymatic oxidation of polyunsaturated fatty acids (PUFA) (Shah, 2005). In plants, linoleic acid (LA; 18:2 $\Delta^{9,12}$) and α-linolenic acid (ALA;18:3 $\Delta^{6,9,12}$) are the principal precursors to a family of oxylipins that include volatile insect attractants, aromas and flavors, cyclized lipid mediators such as the phytohormone jasmonic acid (JA), and reactive products with antimicrobial or phytotoxic properties (**Fig. 2**). An important class of oxylipins related to defense and central to our studies are derived from the initial oxygenation of PUFA catalyzed by 9- and 13-lipoxygenases (9- and 13-LOX). As a result of the different regiospecificities of these LOX's, the fatty acid hydroperoxides thus generated are metabolized by different routes to various classes of oxylipins with consequent structure-activity specificities in biological responses.

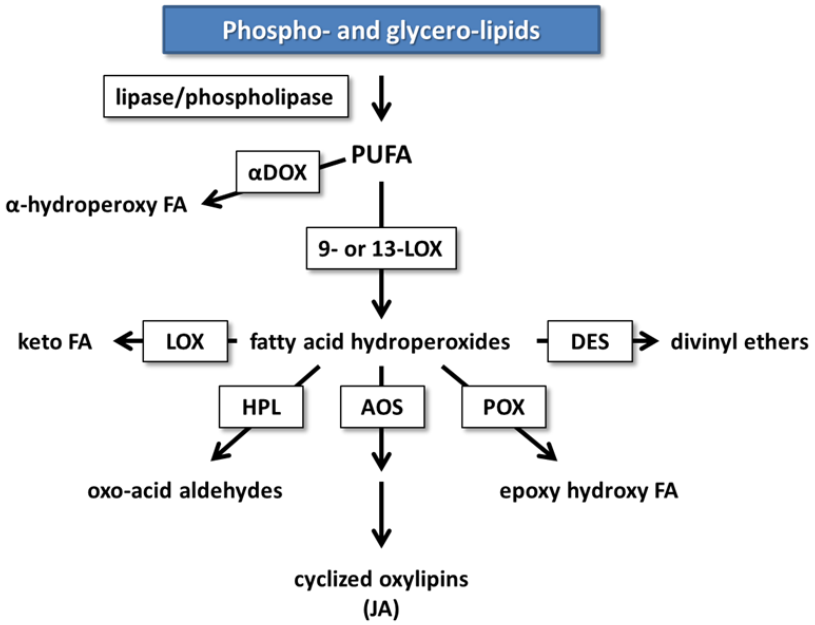


Fig. 2. Major branches of the oxylipin pathway and their corresponding metabolites derived from fatty acid hydroperoxides. AOS, allene oxide synthase; DES, divinyl ether synthase; αDOX, α-dioxygenase; HPL, hydroperoxide lyase; LOX, lipoxygenase; POX, peroxygenase. Not shown is the non-enzymatic, free radical catalyzed oxidation leading to phytoprostane formation and the generation of hydroxy fatty acids by fatty acid hydroxylase or hydroperoxide reductase. [modified from *J. Shah, Annu. Rev. Phytopath* (Shah, 2005)]

Structure-activity studies with PUFA implicate the action of a 9-lipoxyenase (9-LOX) in the initial signal generation from EP that leads to a postulated reactive intermediate(s) (Bostock et al., 1981; Preisig and Kuc, 1985). However, activation of defense responses occurs in a JA-dependent manner in Arabidopsis, indicating additional downstream regulation within the allene oxide synthase (AOS) branch and involvement of 13-LOX (Savchenko et al., 2010). There are important unresolved issues about EP action in crop plants such as tomato (*Solanum lycopersicum*) that can now be addressed with sequenced genomes and technical advances in transcriptomics, oxylipin profiling, and rapid/high-throughput methods for functional analyses. For example, we lack definitive evidence for a role of specific LOX isoform(s) as well as other enzymes in oxylipin branch pathways in EP action. Genes for many of these steps have now been identified and can be functionally evaluated in transient assays and in stably transformed plants.

The following observations about EP elicitor activity, derived largely from studies in Solanaceous plants, provide a foundation for our current investigations.

- Optimal activity is associated with C-20 fatty acids with all *cis*-unsaturation at Δ5,8,11,14 (AA) or Δ5,8,11,14,17 (EPA), and AA and EPA are equivalent in elicitor activity (Bostock et al., 1981; Preisig and Kuc, 1985).
- A free carboxyl is essential and non-hydrolyzable esters of AA are inactive as elicitors, features that meet the free fatty acid substrate requirement of plant LOXs (**Fig. 3**).
- 5-Hydroperoxyeicosatetraenoic acid (5-HpETE) and other LOX metabolites of AA are formed within 10 minutes of AA treatment of potato tissue (Ricker and Bostock, 1994).
- The critical role for plant 9-LOXs in the activation of EP is suggested i) by structure-activity studies with related fatty acids (Bostock et al., 1981; Preisig and Kuc, 1985); ii) by experiments with LOX-null potato cultures; and iii) by studies with LOX inhibitors (Bostock et al., 1986; Preisig and Kuc, 1987).
- AA and/or metabolites of AA are released from spores into host tissue within 9-12 hr after inoculation, concomitant with the onset of biochemical responses to infection (Ricker and Bostock, 1992).
- LOX, phospholipase and lipase are activated following infection of potato with *P. infestans* (Bostock, 1989), and LOX activity is induced in leaves and tubers after treatment with AA (Bostock et al., 1992; Fidantsef and Bostock, 1998).
- EP induce very specific and immediate changes in isoprenoid biosynthesis, redirecting the flow of carbon from sterols and steroid glycoalkaloids to sesquiterpenoid phytoalexins. These changes are evident at the level of gene expression for specific isoforms of HMG-CoA reductase and for activities later in the pathway at the level of farnesyl-PP (Tjamos and Kuc´, 1982; Zook and Kuc, 1991; Choi et al., 1992).

Fig. 3. Classic assays for EP elicitor activity in potato tuber disks and uptake and toxicity in detached leaves. Images taken 72-96 h after treatment. (Bostock et al., 1981)

- EP also induce lipid peroxidation, ethylene, phenylalanine ammonia-lyase, lignin, and peroxidase in the plant (Bostock et al., 1986).
- EP and their LOX products elicit programmed cell death (Wang et al., 1996; Knight et al., 2001), reactive oxygen species (ROS; (Yoshioka et al., 2001), and induce resistance in members of the Solanaceae, pearl millet, and Arabidopsis against various pathogens (Rozhnova et al., 2003; Amruthesh et al., 2005; Savchenko et al., 2010).
- Highly purified branched β-1,3-glucans, major carbohydrate polymers in oomycetes that function as MAMPs in some plants (Boller and Felix, 2009), do not have inherent elicitor activity in potato, yet dramatically enhance sensitivity to EP by up to several orders of magnitude (Bostock et al., 1982; Preisig and Kuc, 1985).
- The phytohormone abscisic acid (ABA), which induces susceptibility in plants to *Phytophthora* spp. and other pathogens (Henfling et al., 1980; Dileo et al., 2010), suppresses EP-triggered immunity (Bostock et al., 1982).

These and other observations suggest the model depicted in **Figure 4** for EP-triggered immunity in oomycete-plant interactions.

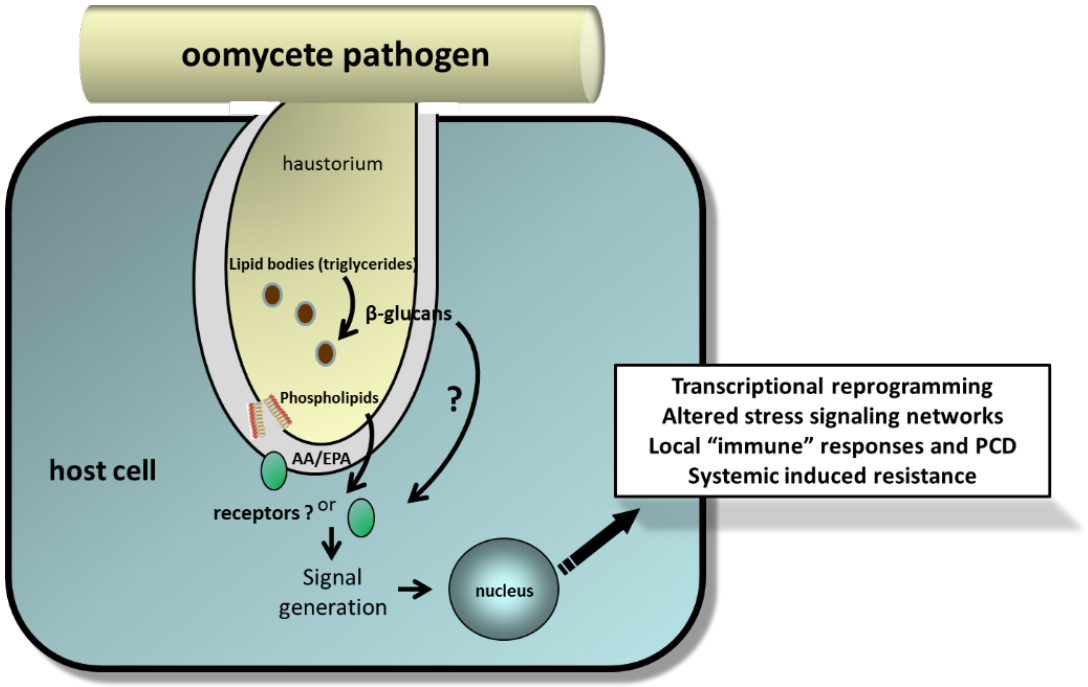


Fig. 4. General model for EP-triggered immunity during infection of plants by oomycete pathogens.

Oxylipins in disease and defense.

The role of lipids as important determinants in plant-microbe interactions is now well-established, with many examples of bioactive lipids of pathogen and plant origin (Shah, 2005; Kachroo and Kachroo, 2009; Koo and Howe, 2009; Walley et al., 2013), including potential lipid receptors such as phosphatidylinositol-3-phosphate that may participate in the uptake of oomycete effectors by the host cell (Kale and Tyler, 2011). There are a number of studies that have shown the association of oxylipin signaling in HR-associated cell death and immunity, but these have focused on oxylipins derived from endogenous plant PUFA such as LA and ALA (Veronesi et al., 1996; Rance et al., 1998; Weber et al., 1999; Itoh and Howe, 2001; Fammartino et al., 2007; Boeglin et al., 2008; Andreou et al., 2009; Koo and Howe, 2009; Mosblech et al., 2009; Fammartino et al., 2010). The role of 9-LOX and 9-LOX metabolites derived from LA and ALA following injury to the plant is well illustrated in the interaction of tobacco and *Phytophthora parasitica* var. *nicotianae.* Here, the disease phenotype is dramatically altered by silencing or overexpression of *NtLOX1* and of *NtDES1*, which encodes divinyl ether synthase (DES), a CYP74 cytochrome P450 monooxygenase (Fammartino et al., 2007). DES converts 9-hydroperoxides of LA and ALA to their 9-divinyl ethers, such as colneleic acid, which was first discovered in potato (Galliard et al., 1973). Divinyl ethers have antimicrobial activity and occur in late blight-infected potato and in TMV-infected tobacco leaves during local lesion development (Weber et al., 1999), suggesting a role in defense. DES is encoded by a single gene in tomato, and its cDNA has been constitutively expressed and characterized (Itoh and Howe, 2001).

Ongoing and recent work on EP signaling

In spite of the intriguing features of EP activity in plants, there has been relatively little research to resolve EP mode of action, in part because of historical limitations of experimental systems and gaps in our understanding of plant oxylipin pathways. However, improved analytical methods, the discovery of new oxylipins, and the characterization of key branch points in biosynthesis have advanced understanding of the metabolism of fatty acid hydroperoxides and provided reagents and materials to study them. Our discovery that Arabidopsis (Col-O) responds to AA and that Arabidopsis plants engineered to express very low but detectable levels

of eicosapolyenoic acids ("EP plants") have remarkably altered phenotypes to biotic challengers has sparked renewed interest and opportunities for contemporary approaches (Qi et al., 2004; Savchenko et al., 2010).

EP plants, in which the fraction of EP within the total fatty acid composition of Arabidopsis is less than 1 mole %, are morphologically indistinguishable from wild-type, but display enhanced resistance to *P. capsici*, *Botrytis cinerea*, and aphids. However, EP plants are more susceptible to *Pseudomonas syringae* pv. *tomato* (DC3000) (Savchenko et al., 2010). The differential effect of EP on disease and pest outcomes corresponds to EP's differential impacts on the SA and JA signaling networks, an effect that is dependent upon JA as demonstrated with a JA-deficient *aos* mutant line. AA, but not "non-MAMP" fatty acids (e.g., LA, 18:2 $\Delta^{9,12}$; ALA, 18:3 $\Delta^{9,12,15}$), specifically triggers defense network responses in Arabidopsis leaves without inducing necrosis. Six LOX isoforms of Arabidopsis (*AtLOX1-6*) also respond differentially to the presence of AA, suggesting a degree of specificity at this level.

Microarray analysis of Arabidopsis roots inoculated with *P. capsici* reveal eight-fold induction 16 hours after inoculation of a small network of stress responsive genes (unpublished; (Obayashi et al., 2009)). These coexpressed genes include isoforms of an mRNA deadenylase, *AtCAF1a* and *AtCAF1b*, as well as a negative regulator of ethylene biosynthesis (Li et al., 2011), and a gene encoding a small protein of unknown function, here designated Small Up-regulated Protein (SUP). *AtCAF1a* and *AtCAF1b* are rapidly and transiently induced by wounding and regulate susceptibility to oxidative and salt stress (Walley et al., 2010). The CCR4-CAF1 complex catalyzes mRNA deadenylation, an important mechanism for regulating gene expression and cellular homeostasis that is conserved among eukaryotes (Walley and Dehesh, 2010). Transcriptome analyses of roots from EP transgenic plants in the absence of the pathogen reveal that *AtCAF1b*, normally transiently induced during stress events, and *SUP* are constitutively expressed, suggesting a novel EP-mediated transcriptional and/or post-transcriptional regulation. The AA-induced expression pattern and known functions of these genes implicates them in a stress response network in Arabidopsis that serves some unique function when the plant encounters AA. The significance of these changes in the transcriptome are unclear, but a comparative analysis of the transcriptome in AA-treated and *P. capsici*-inoculated tomato roots by RNA-Seq should be informative and provide leads concerning EP mode of action.

EP- induced resistance in tomato. Tomato provides an excellent crop model and an experimental system of choice because of its importance in agriculture, the genetic resources available, and clear disease phenotypes with *P. capsici* and other pathogens that can be readily quantified. Although we continue to use Arabidopsis in parallel as a model for its genetic resources applicable to tomato and other crop species, we have developed both hydroponic and soil-based experimental formats with the tomato-*P. capsici* system that enable characterization of EP-triggered immunity within a natural host-pathogen interaction (Dileo et al., 2010)(**Fig. 5**).

Fig. 5. Tomato seedlings (cv. New Yorker) from a hydroponic system four days post inoculation (dpi) with zoospores of *P. capsici* (isolate Yolo-1; 10^4/ml; left panel). Collapsed seedling, three dpi (right panel).

The sodium salts of AA and EPA, but not of LA and ALA, when applied to roots of hydroponically grown tomato seedlings induce resistance to *P. capsici* (Roberts et al., 2012). Tomato seedlings pretreated with EP and then exposed to a strong challenge inoculation 3 days after removal from the inducing treatment show significantly less symptom development and seedling collapse, and are "primed" to respond to the attempted infections by mounting an induced lignification response. The crowns are not directly exposed to the fatty acids during the pretreatment phase. However, following inoculation, the crowns of the protected plants at the air-water interface, although discolored, remain firm and structurally sound. In contrast, seedlings pretreated with Na-LA or Na-ALA prior to inoculation look similar to the H_2O-pretreated, inoculated controls, with extensive softening and dissolution of the host stem and crown tissue.

Analysis and implications of oxylipin gene expression in tomato roots. Although earlier studies are compelling for a significant role of the 9-LOX/DES oxylipin pathway in plant-microbe interactions and induced immunity in the Solanaceae, they do not address the structure-activity

specificity of EP or the strength of the induced resistance observed following EP treatment. We suggest that novel or uncommon oxylipins are generated from EP to alter the course of LA and ALA peroxidative metabolism to provoke the intense plant response and localized necrosis in tomato and potato. Also noteworthy is that DES is present in Solanaceous plants, but not in Arabidopsis, which reinforces our view that EP and other stress regimes will induce distinct oxylipin profiles in Arabidopsis and tomato with possibly different phenotypes.

We recently completed a targeted gene expression analysis of a collection of oxylipin pathway genes in tomato roots. A 9-LOX/9-DES pathway is rapidly and strongly induced in AA-treated tomato roots, with strong induction of *TomLOXE* and *LeDES* transcript accumulation relative to the H_2O-treated control and LA-treated roots. Transcript levels for another 9-LOX (*TomLOXA*) and transcripts for the 9- and 13-AOS genes (*LeAOS3* and *LeAOS*, respectively) were not differentially affected by the treatments.

Radiolabelling studies with ^{14}C-AA indicated rapid formation of various hydroperoxides of AA, incorporation of the applied AA into phospholipids, and formation of uncharacterized polar lipids in oxidized fractions (Preisig and Kuc, 1988; Ricker and Bostock, 1994). With the high resolution and sensitivity of current methods now available to us (Yang et al., 2009), we have begun an oxylipin profiling search for novel divinyl ethers and other oxylipins potentially formed in EP-treated plants. In preliminary experiments, we found that the oxylipin profile in AA-treated tomato roots is very different from that in LA- or H_2O-treated control roots (Robinson et al., 2014). These analyses have revealed that there are novel oxylipins formed in the plant from AA and suggest that the endogenous plant oxylipin profile is altered by the treatment.

Predisposing stress and induced resistance

Brief episodes of root stress such as salinity and water deficit at levels that commonly occur in agricultural systems can predispose plants to pathogens (Bostock et al., 2014). Predisposition as a result of abiotic stress events are well-documented in plant-oomycete interactions, whereby stressed plants succumb to levels of inoculum they would normally resist. The phytohormone abscisic acid (ABA) accumulates rapidly in roots and shoots as an adaptive response to these abiotic stresses, but also can contribute to increase disease susceptibility. Antagonism between SA and ABA is well documented in plant-microbe interactions (Mohr and Cahill, 2007; Jiang et

al., 2010), and ABA antagonizes systemic acquired resistance (SAR) induced by plant activators in *Arabidopsis* and tobacco (Yasuda et al., 2008; Kusajima et al., 2010). In addition, early studies on EP showed that ABA also inhibits AA elicitor activity in potato and induces susceptibility to *Phytophthora infestans* (Henfling et al., 1980; Bostock et al., 1982).

Because of the potential for signaling conflicts in plants exposed to different stresses, we investigated how predisposing root stress impacts induced resistance in tomato with two plant activators that target SA signaling (Pye et al., 2013). 1,2,3-Benzothiadiazole-7-thiocarboxylic acid-s-methyl-ester (BTH) is sold under the trade name Actigard in North America (Syngenta Crop Protection) and used commercially in crop protection against bacterial, fungal and oomycete diseases. Tiadinil (TDL; N-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide), registered in Japan under the trade name, V-GET, also targets SA signaling (Nihon Nohyaku Co., Ltd.), and is typically used as a root dip in rice culture to induce resistance against the rice blast pathogen, *Magnaporthe oryzae*. Using our hydroponic format, we tested the effect of pretreatment of tomato seedlings with TDL and BTH on salt-induced predisposition to the bacterial speck pathogen, *Pseudomonas syringae pv. tomato* (*Pst*), and to *P. capsici*. We found that TDL applied to roots strongly protects leaves from disease caused by *Pst* in both non-stressed and salt-stressed plants. In contrast, neither TDL nor BTH protects roots from *P. capsici*. The protection induced by plant activators against *Pst* does not result from reduced ABA accumulation and, although overall disease is less in both non-stressed and salt-stressed plants by chemically-induced SAR, plant activators do not reverse the salt-induced increment in disease severity.

We also investigated TDL action in SA-deficient NahG plants to see if TDL induces resistance under the different stress regimes in this highly susceptible background. NahG plants were more susceptible to *Pst* and accumulated significantly less SA following *Pst* infection than the WT background 'New Yorker'. However, TDL strongly protected the NahG plants and mitigated any predisposing effect of salt-stress on bacterial speck disease.

Summary

Contemporary approaches for receptor discovery and mode of action are identifying novel targets and additional surveillance options in plants against pathogens. For example, inter-family transfer of the EF-Tu PRR from Brassicaceae (Arabidopsis) to Solanaceae (tomato and tobacco)

increases the spectrum of disease resistance in the recipient hosts, indicating that heterologous expression of PTI recognition systems holds promise as a strategy for more effective and durable disease resistance (Lacombe et al., 2010). Research on EP is advancing knowledge of an understudied class of MAMPs and providing new insights into lipid signaling during plant stress responses. Likewise, we anticipate that determining the basis for EP perception and action in plant-oomycete interactions will identify targets that could be exploited in other crop and landscape species to enhance disease resistance against oomycetes and possibly other attackers. Nonetheless, EP-triggered immunity presents a number of difficult challenges. One of these is that mixed and potentially conflicting messages emanate from phytohormone-regulated response networks following EP treatment to confer the resistance phenotype. For example, at low EP concentrations, the induced resistance is largely mediated by JA/JA-signaling at the expense of SA-mediated responses. However, this may not be the case at higher EP concentrations (Fidantsef et al., 1999). In addition, oxylipin biochemistry is different among different plant species. For example, to our knowledge, in contrast to members of the Solanaceae, Arabidopsis does not produce divinyl ethers from PUFA. Nonetheless, with rich genetic resources and sequenced genomes for both host and pathogen, the tomato – *P. capsici* interaction provides a robust and agriculturally relevant model for determining EP mode of action. The plant and pathogen can be manipulated for biochemical and molecular genetic studies, the disease presents a rapid and quantifiable phenotype, and tomato oxylipin metabolism is well-studied.

Mixed messages are also evident in predisposition where abiotic stress triggers ABA to defeat resistance and inhibit the action of MAMPs such as AA. Although there is evidence for antagonism between ABA and the SA and JA networks, further research is needed to clarify these interactions. Nonetheless, plant activators may partially offset the impact of predisposing abiotic stress. Although our experiments are conducted under highly controlled conditions, we are encouraged by the fact that TDL induces resistance in both salt-stressed and non-stressed plants and in plants severely compromised in SA accumulation. Future research with plant activators should consider their use within different abiotic stress contexts to fully assess outcomes in disease and pest protection.

Acknowledgements

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