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Separation of early and late responses to herbivory in Arabidopsis by changing plasmodesmal function

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Summary

Herbivory results in an array of physiological changes in the host that are separable from the associated physical damage. We have made the surprising observation that an *Arabidopsis* line (*pdko3*) mutated in genes encoding plasmodesmal proteins is defective in some, but not all, of the typical plant responses to herbivory. We tested the responses of plasma transmembrane potential (V_m) depolarization, voltage gated K^+ channel activity, cytosolic calcium $[Ca^{2+}]_{cyt}$ and reactive oxygen species (ROS) (H_2O_2 and NO) release, shoot-to-root signaling, biosynthesis of the phytohormone jasmonic acid (JA) and the elicitation of volatile organic compounds (VOCs). Following herbivory and the release of factors present in insect oral secretions (including a putative β -galactofuranose polysaccharide), both the *pdko3* and wild type (WT) plants showed an increased accumulation of $[Ca^{2+}]_{cyt}$, NO and H_2O_2 . In contrast, unlike WT plants, the mutant line showed an almost complete loss of voltage gated K^+ channel activity and V_m depolarization, a loss of shoot-induced root- V_m depolarization, a loss of activation and regulation of gene expression of the JA defense pathway, and a much diminished release and altered profile of VOCs. The mutations in genes for plasmodesmal proteins have provided valuable genetic tools for the dissection of the complex spectrum of responses to herbivory and shown us that the responses to herbivory can be separated into a calcium-activated oxidative response and a K^+ -dependent V_m -activated jasmonate response associated with the release of VOCs.

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Introduction

Upon herbivory by chewing insects, plants respond with a cascade of events that lead to the activation of defense mechanisms. These include perception of molecular patterns or effectors of defense ([Bos et al., 2010](#); [Bonaventure et al., 2011](#)), elevation of cytosolic calcium ($[Ca^{2+}]_{cyt}$) ([Reddy et al., 2011](#)), plasma transmembrane potential (V_m) depolarization ([Maffei et al., 2004](#)), ion efflux/influx ([Maischak et al., 2007](#)), mitogen-activated protein kinase (MAPK) activation and protein phosphorylation ([Arimura and Maffei, 2010](#); [Arimura et al., 2011](#)), activation of NADPH oxidase and production of ROS ([Maffei et al., 2006](#)), production of ethylene and jasmonate ([Arimura et al., 2009](#)), expression of late defense response genes ([Wu and Baldwin, 2010](#)), and emission of volatile organic compounds (VOCs) ([Baldwin, 2010](#); [Maffei et al., 2011](#)). These events start locally at the feeding site but can spread systemically throughout the plant ([Maffei et al., 2007](#); [Wu and Baldwin, 2009](#)). Although the individual responses that comprise these pathways have been widely catalogued, the connections between them and their interdependence have been little studied.

These pathways have inbuilt capacity for specificity that, for herbivory in particular, allow them to distinguish biotic attack from the physical damage associated with feeding ([Bricchi et al., 2010](#)). In this context, a special role is played by $[Ca^{2+}]_{cyt}$ variations, triggered by oral secretions (OS) associated with herbivore feeding. The fact that single or repeated mechanical wounding alone is not sufficient to elicit significant $[Ca^{2+}]_{cyt}$ variations ([Bricchi et al., 2010](#)) points to oral factors [or herbivore-associated elicitors ([Bonaventure et al., 2011](#))] as triggers for a $[Ca^{2+}]_{cyt}$ burst. The burst is followed by an isotropic wave of V_m depolarization, and ROS production that travels through the attacked leaf ([Maffei et al., 2004](#); [Bricchi et al., 2010](#)). These early events trigger local and systemic responses in the plant body for the production of second messengers that activate target proteins and transcription factors, eventually leading to expression of specific response genes, including those involved in the production of low MW volatiles for, amongst other activities, the

attraction of insect predators ([Wu and Baldwin, 2010](#); [Bonaventure et al., 2011](#); [Karban et al., 2011](#)).

Central to the success of these defenses is the need for local and systemic communication between cells. For plant cells, surrounded by cell walls, symplastic continuity is achieved through the presence of plasmodesmata (PD). These plasma-membrane-lined channels, bridge the cell wall, provide symplastic continuity, and provide soluble and membrane environments for the passage of small and some large molecules ([Maule et al., 2011](#)) and the potential for electrical conduction ([Van Bel and Ehlers, 2004](#)).

Following recent advances in our understanding of the molecular composition of PD ([Amari et al., 2010](#); [Benitez-Alfonso et al., 2010](#); [Faulkner and Maule, 2011](#); [Fernandez-Calvino et al., 2011](#)) we aimed to test the impact of mutations in genes for specific PD proteins with respect to defense signaling in response to herbivory. Altered accumulation of some PD proteins has been shown to alter molecular flux through the channel ([Xu and Jackson, 2010](#); [Maule et al., 2011](#)). For example, reduced expression of members of the PD-located protein (PDLP) family showed increased molecular trafficking from cell to cell ([Thomas et al., 2008](#)). PDLPs are type-I membrane proteins with receptor-like properties, although the nature of any potential ligand is not known ([Amari et al., 2010](#)). Using Arabidopsis plants mutated for *pdlp* genes our data not only implicate PDs directly in defense against herbivory but indicate that some molecular responses to herbivory can be separated genetically from each other and from the overall defense response.

Results

Membrane depolarization in response to herbivory is sensitive to plasmodesmal disruption

When *Spodoptera littoralis* caterpillars were fed on leaves (called ‘herbivore wounding’ or HW) there was both physical damage to the tissues and a physiological disruption caused by the presence of herbivore saliva, which include specific eliciting molecules. In Arabidopsis ([Zebelo and Maffei, 2012](#)) and other plants ([Maffei et al., 2007](#)), these led almost immediately to Vm depolarization, which spread through the rest of the leaf. This effect remained for about 6 h before the leaf recovered over the subsequent 12 h ([Figure 1A](#)). This response was distinct from the effects of mechanical damage (MD) alone (the zero value of [Figure 1A,B](#)) ([Maffei et al., 2004](#)) but the phenomenon could be recapitulated using the application of OS or specific oral factors to MD tissues ([Figure 1B](#)) ([Bricchi et al., 2010](#)).

Figure 1.

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Induced changes in plasma membrane potential (Vm) following herbivory are mediated redundantly by PDLP proteins.

(A) Time course comparison of *Arabidopsis thaliana* WT and the *pdko3* mutant line for *Spodoptera littoralis* (HW)-induced Vm depolarization reveals a significantly ($P < 0.05$) reduced response in the mutant line.

(B) Individual *pdlp* mutant plant lines for six members of the family treated with *S. littoralis* oral secretions (OS) and Fraction α (see also [Figure 2](#)) show variable Vm responses, with none as marked as that seen for the triple mutant *pdko3*. For each time point in (A) and individual mutants

and the WT in (B), at least 10 biological replicates and 30 measurements for each replicate were performed. In both panels, the zero value corresponds to the V_m value of mechanical damage (MD). Error bars represent SEM ($n = 40\text{--}50$); different letters indicate significant ($P < 0.05$), Tukey–Kramer HSD differences.

PDLPs in Arabidopsis are represented by a gene family of eight members whose expression profiles differed widely across different plant tissues but for which many members were expressed to some degree in leaves (Thomas *et al.*, 2008). Previous work showed that removing individual PDLP genes had little impact on PD molecular flux (Thomas *et al.*, 2008), or on the proteins' functions as viral movement protein receptors (Amari *et al.*, 2010), but combinatorial mutants could be defective in both processes. When a *pdlp* triple knock-out mutant (At2g33330, At1g04520 and At5g43980; *pdko3*), which showed no phenotypical differences with respect to the wild type (WT), was used in feeding experiments with *S. littoralis* the same V_m depolarization response did not occur (Figure 1A); a response at only approximately 15% of that of WT plants was established in the first 6 h and retained to 24 h after treatment.

Since PDLPs are receptor-like proteins, we tested whether specific PDLPs may be involved in the perception of herbivory. When individual *pdlp* mutant plants for six members of the family were treated with OS or oral factors, different individual V_m responses were seen (Figure 1B), with none as marked as that seen for *pdko3*. This finding suggested that PDLPs were redundant in their support for transmission of the V_m response.

***Spodoptera littoralis* OS contain specific active fractions**

Receptor-like functions usually involve interactions between the receptor protein and specific chemical ligands. Besides the already characterized herbivore-induced elicitors (Bonaventure *et al.*, 2011), several other compounds resulting from the breakdown of cell walls are known to induce plant cell responses, including V_m variations. In particular, oligosaccharides cause a strong V_m depolarization (Felle *et al.*, 1995, 2000; Thain *et al.*, 1995; Mithöfer *et al.*, 2005). In parallel with the analysis of individual *pdlp* mutant lines, OS were fractionated and further analyzed for the search of possible oligosaccharide elicitors. Separation of acetylated *O*-methyl glycosides from the secretions revealed that the more abundant monosaccharides were galactose and glucose, whereas pentose sugars were present in small amounts (Figure 2A). Methylation analysis aimed at determining sugar branch points revealed the presence of terminal-pentofuranose, 5-substituted-pentofuranose, terminal-glucose, terminal-galactofuranose, 4-substituted-glucose, 6-substituted-glucose, 6-substituted-galactofuranose, and 4,6-substituted-glucose (Figure 2B). Further purification using size exclusion chromatography (SEC) generated 10 fractions (Figure 2C; A–L), which were subjected to bioassay before further analysis. Of 10 fractions, three (A,F,G) induced strong V_m depolarization when applied to MD leaves of WT plants (Figure 3). The major active fraction (FRA) was subjected to complete proteolysis in order to digest all proteins and further separated by SEC into five sub-fractions (Figure 2C; $\alpha\text{--}\epsilon$). Of these, only fraction α (Figure 3; FR α) exerted a significant V_m depolarization on WT plants. Interestingly, this fraction was more active than the effect of either herbivory or OS application (Figure 3; $P < 0.01$). Qualitative and quantitative chemical analyses and $^1\text{H-NMR}$ and $^{13}\text{C}\text{--}^1\text{H}$ heteronuclear single quantum coherence ($^{13}\text{C}\text{--}^1\text{H-HSQC}$) NMR of FR- α revealed the presence of a polysaccharide built up of β -galactofuranose (Figure 2C, inset). The furanose ring and the β -anomeric configuration of galactose were deducible by the low field chemical shift of the carbon anomeric signals (Leone *et al.*, 2010). Studies are underway to purify and characterize this elicitor and the results will be reported soon.

Figure 2.

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Isolation and characterization of the elicitor from *S. littoralis* OS.

(A) Chromatogram of acetylated *O*-methyl glycosides: pentose (peak 1), galactose (peak 2) and glucose (peak 3).

(B) Methylation analysis to determinate branching points: terminal-pentofuranose (peak a), 5-substituted-pentofuranose (peak b), terminal-glucose (peak c) terminal-galactofuranose (peak d), 4-substituted-glucose (peak e), 6-substituted-glucose peak f), 6-substituted-galactofuranose (peak g), 4,6-substituted-glucose (peak h).

(C), Size exclusion chromatography (SEC) of *Spodoptera littoralis* OS. Ten fractions (A–L) were separated and subjected to bioassay before further analysis (see also [Figure 3](#)). Fraction A was subjected to complete proteolysis in order to digest all proteins and further separation by SEC into five sub-fractions (first inset; fractions from α to ϵ). Of these, fraction α (the only one able to exert an effect on Vm) was subjected to chemical analysis and ^1H - and ^{13}C - ^1H heteronuclear single quantum coherence (^{13}C - ^1H -HSQC) NMR and revealed the presence of a β -galactofuranan (downfield shifted carbon signals are indicated by an arrow in the second inset). The furanose ring and the β -anomeric configuration of the sugar were deducible by the low field chemical shift of the carbon anomeric signals.

Figure 3.

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Search for an elicitor of the Vm response that has the potential to be a receptor molecule binding to PDLPs in the WT.

Plasma membrane potential (Vm) depolarization of WT Arabidopsis leaves treated with total OS, fraction A (FRA) or single fractions obtained by SEC (see also [Figure 2](#)). Fractions A, F and G show the same Vm depolarization activity as the total OS, whereas fraction α (FR α , the only deproteinated and purified subfraction of fraction A with an effect on Vm) shows a significantly higher Vm depolarization activity with respect to total OS. Error bars represent SEM ($n = 25$ – 40). The zero value corresponds to the Vm value of the effect of elicitors on mechanical damage (MD). Error bars represent SEM ($n = 40$ – 50); different letters indicate significant ($P < 0.05$, Tukey–Kramer HSD) differences.

Plasmodesmal disruption reduces systemic signaling

In response to herbivory on leaves, plants may rapidly alter physiological conditions (i.e. carbon partitioning patterns) in systemic leaves or roots for later regrowth or reproduction ([Henkes et al., 2008](#)). Hydroponically cultured Arabidopsis provides an ideal tool to study shoot-to-root signaling allowing changes in Vm in the root to be measured in real time following biotic or chemical stress of shoots. When root Vm was measured in WT Arabidopsis, we found that the root Vm was significantly depolarized between 7 and 8 min following either HW, OS or FR α application ([Figure 4](#)). On the other hand, no significant root-Vm depolarization was observed after MD. The *pdko3* mutant showed no root-Vm depolarization after herbivory or the application of either OS or FR α ([Figure 4](#)). As the average distance from leaf stress to the point of Vm measurement in the root was about 13 cm, we have calculated a rate of signal transduction of approximately 110 cm h^{-1} . Published rates of phloem transport range from 30 to 150 cm h^{-1} ([Windt et al., 2006](#)). We then tested whether phloem transport was involved by treating the petiole of the treated leaf with either cold (4°C) or heat (60°C). Both treatments completely abolished the WT HW-, OS- and FR α -

induced root-Vm depolarization ([Figure 4](#)). These data indicate that disruption of PD function reduces systemic signaling and that the HW-induced Vm signal travels through the phloem.

Figure 4.

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Systemic Vm signaling upon herbivory in Arabidopsis WT and the *pdko3* line. Root Vm from hydroponically cultured WT and *pdko3* plants were measured upon treatment of leaves with MD, OS, FR α or after HW. WT root Vm (solid lines) was significantly ($P < 0.05$) depolarized after 7–8 min from treatment, whereas *pdko3* root Vm (dotted lines) did not respond to treatments. Treating WT leaf petioles with either cold (blue line) or heat (red line) blocked root-Vm depolarization in HW treated plants. Error bars represent SEM ($n = 25–40$).

Plasmodesmata separate calcium and ROS signaling responses to herbivory

In the sequence of early and late events in response to herbivory ($[Ca^{2+}]_{cyt}$, Vm depolarization, ROS, and volatile release) ([Maffei et al., 2007](#)), the degree of interdependence has not been easy to dissect. In general, the increase in $[Ca^{2+}]_{cyt}$ precedes membrane depolarization ([Maffei et al., 2007](#)). As *pdko3* showed no significant Vm depolarization in response to treatment with OS or by HW treatment, we tested whether it also showed reduced $[Ca^{2+}]_{cyt}$ and ROS responses, using Calcium Orange as a fluorescent Ca^{2+} probe, and Amplex Red and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM DA) to measure ROS H_2O_2 and NO, respectively ([Figure 5](#)). WT plants responded to MD by a $[Ca^{2+}]_{cyt}$ release that was approximately 10-fold higher than in untreated plants. This increased further with time following HW or application of OS. *pdko3* was also responsive to both MD and the impact of herbivory (OS or HW), although to a lower overall level with respect to WT. The application of FR α showed comparable responses to HW, in both WT and *pdko3*. There was, however, less measurable $[Ca^{2+}]_{cyt}$ even in untreated plants ([Figure 5A](#)). Therefore despite the near absence of a Vm depolarization response in *pdko3*, $[Ca^{2+}]_{cyt}$ still increased following feeding or treatment with OS.

Figure 5.

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Calcium and ROS responses in Arabidopsis WT and the *pdko3* line to *S. littoralis* herbivory (HW), mechanical damage (MD) or application of OS and Fraction α (FR α).

Quantitative data are calculated from confocal analyses for leaves untreated (control), or treated by MD, HW, after 15, 30 or 60 min following OS (MD-OS15, MD-OS30, MD-OS60) or FR α application to MD leaves. cPTIO indicates the addition of the NO scavenger.

(A) Quantitative $[Ca^{2+}]_{cyt}$ variations determined with the Calcium Orange fluorescent dye. MD alone is enough to induce a significant increase of $[Ca^{2+}]_{cyt}$ with respect to controls; however, application of either OS or FR α to MD or herbivory prompt a significant increase of $[Ca^{2+}]_{cyt}$ in WT and to a reduced extent in the *pdko3*.

(B) Quantitative H_2O_2 variations determined with the Amplex Red fluorescent dye. A dramatic increase in H_2O_2 is observed in the *pdko3* following application of both OS and FR α or after HW, with respect to WT. (C), Quantitative NO variations determined with the DAF-FM DA dye. The application of OS and FR α , as well as HW prompt a significant increase in NO concentration with respect to control; however, no significant difference was found between WT and *pdko3*. The effect

of the NO scavenger cPTIO was an almost completed reduction of DAF-FM DA fluorescence. Error bars represent SEM ($n = 8-10$). Different letters indicate significant differences ($P < 0.05$, Tukey–Kramer HSD).

ROS released following HW included H_2O_2 and NO. In contrast to WT Arabidopsis, where H_2O_2 increased in response to MD but more markedly following HW, H_2O_2 showed limited accumulation in *pdko3* in response to MD but an exaggerated response to treatment with OS, FR α or HW (Figure 5B). In contrast, using the fluorescent sensor for NO, the changes in response to MD, OS, FR α application or HW were indistinguishable between the two plant lines (Figure 5C). The specificity of the fluorescence response was confirmed following HW treatment in the presence of the NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethylimidazolinone-3-oxide-1-oxyl [cPTIO; (Leitner *et al.*, 2009)]. Therefore, by using the *pdko3* line these data revealed that, following HW, $[Ca^{2+}]_{\text{cyt}}$ increases and the downstream consequences in electrical signaling and accumulation of different ROS can be separated.

Plasmodesmata are involved in herbivory-induced voltage gated K^+ channels activity

Since herbivore-induced V_m depolarization is not directly dependent on Ca^{2+} variations, we tested the activity of K^+ channels by using the fluorescent indicator FluxORTM. Significantly increased (approximately 3-fold) K^+ channel activity was observed when OS and FR α or herbivory were applied to WT plants, whereas there was only slight increase (<1-fold) in K^+ channel activity, relative to MD treatment, in the *pdko3* line. (Figure 6). Use of the specific inhibitor tetraethylammonium (TEA) significantly reduced the K^+ channel activity of HW WT, although the inhibitor was not able to completely suppress the response (Figure 6).

Figure 6.

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Voltage gated K^+ channel activity. *S. littoralis* herbivory and the application of either OS or FR α induces a strong and significant increase of FluxORTM fluorescence in the WT, with respect to either MD or control.

In the *pdko3* the same treatment induced a significantly reduced effect. The use of the K^+ channel inhibitor TEA partly reduced FluxORTM fluorescence. Error bars represent SEM ($n = 8-10$). Different letters indicate significant differences ($P < 0.05$, Tukey–Kramer HSD). WT = control.

Secondary signaling pathways and their outputs

The ultimate beneficial outcomes of defense response pathways against insects are to deter insect feeding, reduce insect fecundity and/or to attract insect predators. For Lepidopterans the best studied signaling molecule in these pathways is jasmonic acid (JA), which has downstream consequences for the emission of VOCs. From our data relating to the primary signaling responses, we hypothesized that some of these secondary components might be altered in the *pdko3* line, potentially with an impact on insect survival. However, no significant difference was found in *S. littoralis* weight increase when the insect was feeding on either WT or *pdko3*, indicating neither differences in the nutrient value nor any toxicity of *pdko3* plants (Figure S1).

JA and its conjugate (3R,7S)-jasmonoyl-l-isoleucine (JA-Ile) are the products of the JA signaling pathway, their biosynthesis requires activities of allene oxide synthase (AOS) and OPDA-reductase

3 (OPR3) and the intermediate precursor *cis*-(+)-12-oxophytodienoic acid (OPDA). In time course experiments, herbivory on *pdko3* showed reduced accumulations of JA, relative to the WT response (Table 1). Herbivory exerted no significant differences on the JA-Ile content between WT and *pdko3*, although both plants showed significantly higher values with respect to MD. In contrast, OPDA accumulation was always more evident in *pdko3*. The reduced JA response in the *pdko3* line correlated with reduced expression of the genes for the biosynthetic enzymes AOS and OPR3, determined by qRT-PCR (polymerase chain reaction). This contrasted with WT plants where expression of both genes showed significant increases following herbivory (Table 1).

Table 1. JA, JA-Ile and OPDA levels and differential expression of genes involved in JA and VOC production following herbivory (HW) and mechanical damage (MD) in WT and *pdko3*

Compounds	Timing	Specifications			
		WT		<i>pdko3</i>	
		MD	HW	MD	HW
1. Results of compound analysis are expressed as nanogram per gram f. wt; qRT-PCR analysis are shown as fold changes in expression (\pm SEM). In the same row different letters indicate significant ($P < 0.05$) differences.					
2. AOS, allene oxide synthase; OPR3, OPDA-reductase 3; GPS1, Geranyl diphosphate synthase; FPS1, farnesyl diphosphate synthase; CYP82G1, Cytochrome P450 involved in DMNT/TMTT production; TPS03, (E)- β -ocimene synthase/myrcene synthase; TPS04, (E,E)-geranylinalool synthase; TPS21, (-)-E- β -caryophyllene synthase/ α -humulene synthase.					
JA	0'	75.86 (12.64) ^a		98.61 (22.34) ^a	
	30'	99.72 (17.94) ^a	802.19 (85.32) ^b	114.29 (49.22) ^a	356.50 (29.55) ^c
	1 h	172.59 (18.65) ^a	1093.86 (228.32) ^b	181.27 (40.61) ^a	531.45 (120.97) ^c
	2 h	88.63 (10.80) ^a	1030.14 (280.48) ^b	239.85 (45.88) ^c	629.68 (150.19) ^d
	0'	30.33 (22.54) ^a		36.36 (19.76) ^a	
JA-Ile	30'	68.96 (12.86) ^a	318.21 (34.59) ^b	87.01 (25.56) ^a	397.34 (76.62) ^b
	1 h	53.90 (15.28) ^a	320.20 (64.83) ^b	71.81 (32.30) ^a	338.13 (111.72) ^b
	2 h	42.14 (21.34) ^a	230.53 (44.62) ^b	34.30 (8.54) ^a	257.94 (44.45) ^b
	0'	96.46 (32.12) ^a		118.92 (29.87) ^a	
OPDA	30'	189.74 (46.66) ^a	2248.36 (252.02) ^b	379.65 (28.62) ^c	3184.62 (346.20) ^d
	1 h	277.56 (80.28) ^a	2246.67 (237.33) ^b	764.29 (152.75) ^c	3458.54 (151.46) ^d
	2 h	287.99 (56.21) ^a	3819.65 (762.34) ^b	510.33 (117.17) ^c	4614.20 (627.04) ^d
Genes					
	AOS (At5g42650)	1.02 (0.12) ^a	2.04 (0.16) ^b	1.00 (0.08) ^a	-1.46 (0.06) ^c
	OPR3 (At2g06050)	1.02 (0.07) ^a	2.94 (0.46) ^b	1.07 (0.10) ^a	-1.19 (0.14) ^c
	CYP82G1 (At3g25180)	-1.03 (0.04) ^a	2.52 (0.09) ^b	1.61 (0.12) ^c	1.04 (0.01) ^a
	TPS04 (At1g61120)	1.05 (0.08) ^a	4.70 (0.19) ^b	-1.59 (0.07) ^c	-1.21 (0.04) ^d
	GPS1 (At2g34630)	1.08 (0.18) ^a	1.65 (0.30) ^b	1.02 (0.14) ^a	-1.59 (0.03) ^c

Compounds	Timing	Specifications			
		WT		<i>pdko3</i>	
		MD	HW	MD	HW
<i>FPS1</i> (At5g47770)		1.05 (0.14) ^a	1.77 (0.09) ^b	1.13 (0.18) ^a	1.09 (0.04) ^a
<i>TPS03</i> (At4g16740)		1.02 (0.07) ^a	1.92 (0.15) ^b	2.00 (0.38) ^b	1.52 (0.07) ^c
<i>TPS21</i> (At5g23960)		1.03 (0.12) ^a	3.14 (0.17) ^b	-1.11 (0.05) ^c	3.50 (0.23) ^b

Similar gene expression studies were carried out for two enzymes involved in the synthesis of VOCs: geranyl diphosphate synthase, (CYP82G1), and *E,E*-geranylinalool synthase (TPS04) (Arimura *et al.*, 2009). The products of these activities have been shown to be induced by JA and produced upon herbivory in several plant species (Maffei *et al.*, 2011). Expression of these genes paralleled the situation for the JA-biosynthetic genes; expression of both genes was increased following HW relative to MD, but only in the WT line, and the same trend was found for other genes involved in terpenoid synthesis (Table 1). We predicted, therefore, that the *pdko3* line would produce reduced amounts of VOC following herbivory. GC-MS analysis of the vapor phase from the head-space above plants treated by HW showed that *pdko3* plants produced only approximately 20% of the total complement of VOCs detected from treated WT plants (Table 2). Qualitative and quantitative analysis of individual VOCs showed there to be dramatically reduced amounts of specific VOCs in *pdko3* line, with the exception of limonene; however, control WT and *pdko3* plants showed not significant variations (Table 2).

Table 2. VOC emission from Arabidopsis WT and *pdko3* in controls and upon *S. littoralis* herbivory

Compound	WT		<i>pdko3</i>	
	Control	HW	Control	HW
1. GC-MS analyses of plant head-space volatiles show quantitative and qualitative differences between WT and <i>pdko3</i> plants. Compound values are expressed as nanogram per gram fr. wt. (SEM; $n = 3-5$). In the same row, different letters indicate significant differences ($P < 0.05$, Tukey–Kramer HSD).				
Limonene	0.15 (0.01) ^a	0.39 (0.05) ^b	0.18 (0.01) ^a	1.46 (0.21) ^c
<i>O</i> -cymene	0.43 (0.03) ^a	2.32 (0.22) ^b	0.34 (0.02) ^a	0.39 (0.04) ^a
Nonanal	0.22 (0.02) ^a	2.27 (0.34) ^b	0.19 (0.01) ^a	0.23 (0.03) ^a
DMNT	0.31 (0.01) ^a	9.14 (0.28) ^b	0.27 (0.01) ^a	0.35 (0.03) ^a
Pulegone	0.58 (0.04) ^a	2.99 (0.27) ^b	0.55 (0.03) ^a	0.66 (0.04) ^a
3-eicosene	0.63 (0.06) ^a	4.90 (0.51) ^b	0.60 (0.05) ^a	0.69 (0.07) ^a
Butyl-octanol	2.02 (0.21) ^a	6.27 (0.58) ^b	1.83 (0.17) ^a	1.91 (0.21) ^a
Methyl-dodecanol	1.23 (0.11) ^a	4.07 (0.40) ^b	1.10 (0.20) ^a	1.33 (0.18) ^a
Methyl-dodecane	1.35 (0.18) ^a	4.73 (0.31) ^b	1.47 (0.29) ^a	1.40 (0.20) ^a
Longifolene	0.77 (0.09) ^a	3.04 (0.38) ^b	0.66 (0.08) ^a	0.61 (0.06) ^a
β -caryophyllene	0.12 (0.01) ^a	1.23 (0.14) ^b	0	0
β -elemene	0.11 (0.02) ^a	2.14 (0.29) ^b	0.10 (0.02) ^a	0.12 (0.01) ^a
Aromadendrene oxide	0.17 (0.03) ^a	12.11 (1.09) ^b	0.14 (0.02) ^a	0.15 (0.01) ^a
<i>Trans</i> - β -ionone	2.27 (0.23) ^a	19.25 (2.01) ^b	2.28 (0.31) ^a	2.45 (0.33) ^a
Unknown methyl-alkane	1.45 (0.15) ^a	5.02 (0.68) ^b	1.41 (0.23) ^a	1.62 (0.18) ^a
TMTT	1.87 (0.24) ^a	4.58 (0.42) ^b	1.98 (0.32) ^a	2.15 (0.14) ^a

Compound	WT		<i>pdko3</i>	
	Control	HW	Control	HW
Unknown methyl-alkane	1.24 (0.38) ^a	8.64 (0.01) ^b	1.16 (0.15) ^a	1.23 (0.13) ^a
Total content	14.91 (1.33) ^a	93.10 (8.72) ^b	14.25 (0.97) ^a	16.74 (1.17) ^a

Discussion

We have made the surprising observation that an Arabidopsis line (*pdko3*) mutated in genes encoding PD proteins is defective in some, but not all, responses to insect herbivory. Following HW and the release of OS, similar to WT plants *pdko3* shows a release of $[Ca^{2+}]_{cyt}$, NO and a release of H_2O_2 . Unlike WT plants, *pdko3* shows an almost complete loss of the Vm depolarization response which correlates with a reduced voltage gated K^+ channel activity, reduced activation of the JA defense pathway, and a much diminished release and altered profile of volatile chemicals.

Host perception of invading pathogens/pests is achieved through recognition of either their innate molecular patterns (e.g. surface molecular features) or their effector molecules released to manipulate host defenses ([Anderson et al., 2010](#); [Alba et al., 2011](#)). For chewing herbivores, where MD alone is insufficient to elicit the range of responses, little is still known about the nature of such elicitors ([Bonaventure et al., 2011](#); [Mori and Yoshinaga, 2011](#)). Elicitors such as peptides, β -glucans and other oligosaccharides derived from the breakdown of cell walls may induce Vm depolarization ([Arimura et al., 2011](#)). Using the differential responses of *pdko3* and WT lines as an assay, biochemical separation of OS identified a highly efficacious fraction able to elicit a K^+ -dependent Vm response, containing a polysaccharide made of β -galactofuranose; the full chemical characterization is underway and will be published soon.

Insect feeding and isolated insect-derived elicitors are known to lead to Ca^{2+} signatures ([Maffei et al., 2007](#); [Arimura and Maffei, 2010](#)) and these events have been associated to Vm depolarization ([Maffei et al., 2004](#)). As *pdko3* showed $[Ca^{2+}]_{cyt}$ induction we can conclude that $[Ca^{2+}]_{cyt}$ might not be directly involved in Vm depolarization. Rather the induced Vm depolarization appears to be associated to an increased voltage gated K^+ channel activity. In Lima bean H_2O_2 has been shown to trigger calcium signatures and cause Vm changes ([Maffei et al., 2006](#)). However, ROS appear not to be involved in Vm depolarization, as the dramatic increase of H_2O_2 shown by *pdko3* upon HW or OS was not correlated to any alteration in the plasma membrane ion balance.

Potassium represents the major osmotically active cation in plants cells and is fundamental for plant functions such as control of the membrane potential ([Lebaudy et al., 2007, 2008](#); [Geiger et al., 2009](#)). Some K^+ channels are activated by calcium sensing proteins (e.g. CBL1/9) that play a key role in decoding calcium transients ([Batistic and Kudla, 2009](#)). Upon herbivory, the increased concentration of cytosolic Ca^{2+} triggers some inward K^+ channels that eventually cause the plant cell Vm depolarization. In the *pdko3* it appears that the connection between increased calcium and K^+ channel activation is broken leading to significantly decreased Vm depolarization, although the lower absolute levels of $[Ca^{2+}]_{cyt}$ in *pdko3* leaves open the possibility of a threshold effect in K^+ channel activation.

JA has been repeatedly shown to be the most important mediator of plant-herbivore interactions and to be responsible for VOC activation ([Baldwin, 2010](#); [Dicke and Baldwin, 2010](#); [Occhipinti et al., 2011](#)). In *pdko3*, the reduced levels of JA and expression of JA-biosynthetic genes, and genes related to terpenoid synthesis correlate with the lack of herbivore response and VOC emission. As the lack of Vm response in the *pdko3* was significantly and positively correlated to the reduction of VOC emission, JA biosynthesis and gene expression, the *pdko3* line appears therefore to dissect

genetically the responses to herbivory into a calcium-activated oxidative response and a K^+ /Vm-activated JA response associated with the release of VOCs. In the laboratory, the consequences of these changes for herbivore survival appear to be benign; this may well differ in the natural environment where the benefit to the plant of VOC release (e.g. as predator attractants) may be more profound ([Maffei et al., 2011](#)). It is interesting to note that the flux of precursors through the JA biosynthesis pathway is similar between WT and *pdko3* plants and that the conversion of OPDA to JA is actually affected in the mutant (without affecting JA-Ile levels). Thus, it seems that the effect of *pdko3* on the JA biosynthesis pathway affects mainly enzymes downstream of OPDA formation.

A secondary question related to the main conclusion to this work is why altering PD function may prevent Vm depolarization and downstream effects? PD provides symplastic connections between living cells that are regulated with respect to molecular flux through the channel by changes in callose (β -1,3-glucan) deposition in the near-cell wall. *pdko3* contains mutations in three members of the PDLF family of PD proteins that have apoplastic ‘receptor-like’ domains, single transmembrane domains and short C-terminal symplastic domains. It has been assumed ([Thomas et al., 2008](#)) that activation of these proteins in the apoplast triggers a signaling pathway involving protein partners in the membrane or symplast resulting in altered PD function. It has been shown ([Thomas et al., 2008](#); [Amari et al., 2010](#)) that the protein family members act redundantly with respect to changing flux through the channel; the redundant nature of PDLF function was confirmed for HW responses in this work. Attempts to assess symplastic connectivity before and after treatment with *S. littoralis* OS, after loading low-molecular-weight dyes around a mechanically damaged site in leaves have so far been unsuccessful. We do not yet understand therefore how changes in PD function impact the host response to herbivory but this behavior of otherwise phenotypically normal plants supports the view that PD function must be playing an important role.

Changes in PD function in *pdko3* abolished systemic Vm depolarization, as shown by the shoot-to-root signaling experiments. The same effect was seen following thermal disruption of phloem transport pointing to a mobile signal exploiting the PD and phloem transport pathways to access remote organs of the plant. The nature of this mobile signal remains unknown.

This work has revealed a surprising correlation between factors influencing symplastic connectivity and downstream consequences of the Vm membrane depolarization response and its propagation through the tissues. While we do not yet know how PD and Vm are functionally linked, the *pdko3* line has provided a valuable genetic tool that has allowed the complex spectrum of response to herbivory to be dissected. The recent finding that the Arabidopsis PDLF5 is essential for conferring enhanced innate immunity against bacterial pathogens in a salicylic acid-dependent manner ([Lee et al., 2011](#)), confirms the major role of the regulation of PD closure as a crucial part of coordinated control of cell-to-cell communication and defense signaling.

Experimental Procedures

Plant and animal material

Arabidopsis thaliana L. (Columbia 0) plants were grown at 22°C from seed in a plastic pots with sterilized potting soil held at 60% humidity, with daylight fluorescent tubes ($120 \mu\text{mol m}^{-2} \text{sec}^{-1}$) and a photoperiod of 16 h. The *pdko3* mutant line was generated by standard genetic crosses as described earlier ([Amari et al., 2010](#)). All experiments were carried out using 20–22-day-old plants [phases III of development ([Boyes et al., 2001](#))]. Hydroponic cultures were assessed in Araponics hydroponic growing system (<http://www.araponics.com/>), seedlings were grown in liquid medium. Plants were supplied with 0.12 ml L^{-1} FloraGro and FloraMicro (GHE,

<http://gb.eurohydro.com/floraseries.html>) dissolved in tap water. The pH was adjusted to 6.0 and root aeration was supplied by an air-pump (Resun, AC-2000) equipped with a mineral sand air stone.

Spodoptera littoralis Bois. (Lepidoptera, Noctuidae) larvae were fed on artificial diet composed of 125 g bean flour, 2.25 g ascorbic acid, 2.25 g ethyl 4-hydroxybenzoate, 750 μ l formaldehyde, 300 ml distilled water and 20 g agar previously solubilized in 300 ml of distilled water. The ingredients (Sigma-Aldrich, <http://www.sigmaaldrich.com/>) were mixed with a blender and stored at 4°C for not more than 1 week. With the exception for VOC collection (see below), plants were fed for 2 h with third instar larvae reared from egg clutches in Petri dishes (6 cm diameter) in a growth chamber with 16 h photoperiod at 25°C and 60–70% humidity. The amount of herbivore damage was kept to 30% of leaf surface as detected by ImageJ image analysis ([Bricchi et al., 2010](#)). Feeding experiments were always performed between 1 and 3 p.m.

Collection of oral secretions

In order to evaluate the effect of *S. littoralis* OS and isolated oral factors (OF), 5-day-old larvae were allowed to feed on Arabidopsis WT leaves for 24 h. Regurgitation was caused by gently squeezing the larva with a forceps behind the head. OS was collected into glass capillaries connected to an evacuated sterile vial (peristaltic pump). Secretions were stored at –20°C until analysis. Five microliters of OS in 5 mM Mes-NaOH (pH 6.0) were applied at the site of MD with a microsyringe and the V_m of leaves was analyzed after 2 h. The OS quantity was assessed after several trials (from 0.5–10 μ l) and was found the most appropriate to obtain reproducible experiments.

Membrane potential determination

Membrane potentials were determined in leaf segments. The transmembrane potential (V_m) was determined with glass micropipettes with a tip resistance of 4–10 M Ω and filled with 3 M KCl as described earlier ([Maffei et al., 2004](#)). Based on topographical and temporal determination of V_m performed previously the electrode was inserted between 0.5 and 1.5 mm from the wounded zone, where a significant V_m depolarization occurs after HW. In order to assess the shoot-to-root signaling, hydroponic cultured plants were placed with the root tip immersed in the V_m measuring well. V_m were measured in the root elongating zone. MD, HW, OS and fraction α were applied on leaves at time 0 and the V_m was continuously measured in roots. Alternatively, the leaf petiole was treated with either cold (4°C) or heat (60°C) stress. Cold stress was provided by a thin hollow tube looping around the petiole. A cold (0°C) salty solution was circulated inside the tube by means of a peristaltic pump and the petiole temperature was assessed with a thermal couple and stabilized to 4°C 20 min prior HW, OS or FR α application, then the temperature was maintained throughout the experiment. For heat treatment we followed the same methodology, but the circulating liquid was kept at 60°C. The results of all V_m measurements are shown as the average number of at least 50 V_m measurements.

Gas chromatography analyses of *S. littoralis* OS saccharides

Monosaccharide composition of non-fractionated OS was analysed by acetylated *O*-methyl glycosides method as reported earlier ([De Castro et al., 2010](#)). Branching points monosaccharides were examined by synthesis of partially methylated alditol acetates (PMAA) ([De Castro et al., 2010](#)). All data suggested the presence of two main sugar residues, glucose and galactose; this latter was essentially present as 6-substituted furanose ring derivative(s).

Saccharide derivatives were then analyzed by GC/MS with an Agilent 6850 and Agilent 5973 Network spectrometer (Agilent Technologies, Inc., <http://www.home.agilent.com>). Instrumental data were: column mod. RTX-5 (30 m × 0.25 mm; Restek Corporation, <http://www.restek.com/>). The carrier gas was He with a 1 ml min⁻¹ flow, injection volume 1 µl in acetone, injection port 250°C with 1:10 split ratio. Solvent delay was 4 min and timetable: 150°C 3 min, 3°C min⁻¹ up to 300°C, 300°C 10 min in 63 min and EI spectrometer range: 40–650 *m/z*. Identification of *O*-methyl glycosides derivatives and PMAA products was made by comparing of both retention time and specific electronic impact fragmentation pattern of standard molecules ([Leone et al., 2010](#)).

Size exclusion chromatography (SEC) and partial purification of OS

Chromatographic purification of molecular compounds in OS was carried out by SEC. Lyophilized samples (7 mg) were dissolved with 1 ml of ammonium bicarbonate (AMBIC) buffer 50 mM. First SEC purification step has been performed applying the sample on a BioGel P2 resin (Bio-Rad, <http://www.bio-rad.com/>) (1.5 × 98.5 cm) and was performed in triplicate to confirm elution profile. Second purification step of fraction A was made on a BioGel P10 resin (Bio-Rad) (2.0 × 41 cm). Elution was with AMBIC buffer 50 mM and 12 ml h⁻¹ flow through a peristaltic pump and a refractive index detector (attenuation 20 mV). Fractions of 2.5 ml for P2 SEC and 2 ml for P10 SEC were collected and combined on the base of chromatogram signal. After chromatographic separation, samples were lyophilized for further analyses.

Protease digestion of fraction A

Fraction A (1.49 mg) from partially purified OS from P2-SEC was digested with protease type XIV (Prod. No. P5147; Sigma-Aldrich) to remove protein contamination. Lyophilized fraction A was dissolved in 900 µl MilliQ water (Millipore, <http://www.millipore.com/>), a proper amount of 10× digestion buffer (Tris–HCl 1 M, NaCl 0.5 M, MgCl 0.1 M, pH 7.5) and 0.5 mg of protease powder were added. Digestion was held for 20 h at 56°C. Before P10-SEC, sample was lyophilized before dissolving in AMBIC buffer.

NMR analyses

¹H and gHSQC spectra were recorded in deuterated water (D₂O) and acquired on a Bruker 600 MHz equipped with a cryo probe, operating at 298 K. gHSQC was measured using standard Bruker software: 512 FIDs of 2048 complex points were acquired with 50 scans per FID; processing and analysis of data were performed with Bruker TopSpin 2.1 program (<http://www.bruker.com/>). The furanose ring and the β-anomeric configuration of galactose were deduced by the low field chemical shift of the carbon anomeric signals ([Leone et al., 2010](#)).

Determination of intracellular calcium variations using confocal laser scanning microscopy (CLSM) and calcium orange

Calcium Orange dye (stock solution in DMSO; Molecular Probes, Invitrogen, <http://www.invitrogen.com>) was diluted in 5 mM MES-Na buffer (pH 6.0) containing 0.5 mM calcium sulphate and 2.5 µM dichlorophenyldimethylurea (DCMU) (Sigma-Aldrich) to a final concentration of 5 µM. This solution was applied on Arabidopsis WT and *pdko3* leaves attached to the plant as detailed elsewhere ([Bricchi et al., 2010](#)). Five micromolar of Calcium Orange solution (about 45 µl) was applied and after 60 min the leaf was mounted on a Nikon Eclipse C1 spectral CLSM stage (<http://www.nikon.com/>) without separating the leaf from the plant in order to assess the basic fluorescence levels as a control. The microscope operates with a krypton/argon laser at 488 nm with a BP of 500–540 nm and a LP of 650 nm. Images generated by the FluoView software

were analyzed using the NIH image software as described earlier ([Mithöfer et al., 2009](#)). After MD, HW and application of fraction α , leaves were perfused (i.e. the solution was delivered to tissues as a liquid phase with a syringe) with Calcium Orange and analyzed by CLSM as described above. Time course experiments included measurements 15, 30 and 60 min after treatment of MD leaves with OS. Measurements were repeated at least five times (biological replicates) and were performed 0.5–1.5 mm from the wounded zone.

CLSM localization of H₂O₂ using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red)

The Amplex Red Hydrogen Peroxide Assay (Molecular Probes) was used for the detection of H₂O₂. Arabidopsis WT and *pdko3* leaves from intact plants in pots were incubated with a 50 μ m Amplex Red solution (in 5 mm Mes-Na buffer, pH 6.0, containing 0.5 mm calcium sulphate and 5 μ m DCMU) as reported earlier ([Maffei et al., 2006](#)). The Nikon Eclipse C1 spectral CLSM was operated with an Ar-Laser (458 nm/5 mW; 476 nm/5 mW; 488 nm/20 mW; 514 nm/20 mW), a HeNe-Laser (543 nm/1.2 mW) and a HeNe-Laser (633 nm/10 mW). After MD, HW and application of fraction α , leaves were perfused with Amplex Red and analyzed by CLSM as described above. Time course experiments and measurements were as above.

CLSM determination of nitric oxide with 4,5-diaminofluorescein diacetate (DAF-FM DA)

To analyze NO accumulation by fluorescence microscopy, non-detached Arabidopsis WT and *pdko3* leaves were gently placed on a glass slide and incubated for 1 h with 50 μ l of loading buffer [10 μ m DAF-FM DA (Molecular Probes) in 5 mm Mes-Na buffer, pH 6.0]. Thereafter the leaf was mounted on a Nikon Eclipse C1 spectral CLSM stage without separating the leaf from the plant. In these experiments an argon laser with an excitation wavelength of 488 nm was used. Emissions were recorded using a 508–525 nm band pass filter. Carboxy-2-phenyl-4,4,5,5-tetra-methylimidazolinone-3-oxide-1-oxyl (cPTIO) (Sigma), an NO scavenger, was dissolved in DMSO and used at final concentration of 1 mM. The treated leaves were perfused with cPTIO as described above before being stained with DAF-FM DA. Leaves were stained with DAF-FM DA after MD, HW and application of fraction α and analyzed by CLSM as described above. Time course experiments and measurements were as above.

CLSM localization of voltage gated K⁺ channels using FluxOR™

Voltage gated K⁺ channels were assayed by using the FluxOR™ potassium ion channel kit from Invitrogen (Molecular Probes). Non-detached Arabidopsis WT and *pdko3* leaves were gently placed on a glass slide and incubated in the dark for 1 h with 100 μ l of loading buffer (deionized water, FluxOR™ assay buffer and probenecid) by following the manufacturer's instructions. Plants were treated with MD, HW and fraction α as above and just before observation 50 μ l of stimulus buffer (deionized water, FluxOR™ chloride-free buffer, K₂SO₄ and Tl₂SO₄) were added by following the manufacturer's instructions. CLSM fluorescence was assayed by using an argon laser with an excitation wavelength of 488 nm was used. Emissions were recorded using a 520–535 nm band pass filter. Twenty millimolar Tetraethylammonium (TEA; Sigma) a K⁺ channel inhibitor, was also used and incubated along with the loading buffer components. Time course experiments and measurements were as above.

Extraction and determination of JA, JA-Ile and OPDA

Arabidopsis WT and *pdko3* leaves were fed by *S. littoralis* and leaves were collected after 30 min, 1 and 2 h of treatment, placed immediately in liquid nitrogen and kept at -80°C until use for extraction as early reported (Occhipinti *et al.*, 2011). The content of JA, JA-Ile and OPDA was determined by comparing retention times and mass spectra of standard solutions with a linear gradient in RP-chromatography (Luna C18, 3.0×150 mm, $3.0 \mu\text{m}$; Phenomenex, <http://www.phenomenex.com/>) and further analysis with a 6330 Agilent Ion Trap LC/MS system. H₂-JA (TCI-Europe) was used as internal standard and precursor ions were detected in negative mode by multiple reaction monitoring (MRM): [M-H]⁻ 209.0, 211.0, 322.0, and 291.0 for JA, H₂-JA, JA-Ile, and OPDA, respectively. The resulting amount of JA, JA-Ile and OPDA was referred to the total fresh weight of leaves (Occhipinti *et al.*, 2011). The reported data are the mean values of at least three biological replicates and several technical replicates.

Collection of plant volatiles, gas chromatography and mass spectrometry

Experiments were conducted in 4 L glass desiccators by using non-flowering Arabidopsis WT and *pdko3* intact plants, placed in 100 ml aluminum containers. Seven plants per single desiccator were used. Plants were illuminated with fluorescent light bulbs generating about $150 \mu\text{mol m}^{-2} \text{sec}^{-1}$ with a photoperiod of 16 h, the temperature inside desiccators was about 24°C and the relative humidity about 70%. Glass desiccators were connected to a GC-grade air generator (HPZA-3500-220; Parker Balston, <http://www.labgasgenerators.com/>) through a cork plug with two openings allowing gases to go in and out. Air was pumped into the jars at a flow rate of 300 ml min^{-1} as previously described (Zebelo *et al.*, 2011). VOCs were sampled with a Carboxen/Polydimethylsiloxane (CAR/PDMS, Sigma-Aldrich) Supelco (Bellefonte, PA, USA) solid-phase micro-extraction (SPME) fiber (model 57334-U). Before use, SPME fibers were always conditioned at 250°C , according to manufacturer's instructions.

Undamaged plants, leaves subjected to MD with a pattern wheel (Bricchi *et al.*, 2010) and plants infested for 6 h were assayed for VOC emission. All experiments were standardized at 6 h, because the presence of eight herbivores for 6 h was found to cause about 30% of leaf damage. Unwounded plants emitted very low levels of VOCs.

SPME fibers were desorbed and VOCs analyzed by gas chromatography–mass spectrometry (GC-MS 6890N-5973A; Agilent Technologies) as previously described (Zebelo *et al.*, 2011). Separated compounds were identified by pure standard comparison, by comparison of their mass spectra and retention indexes (Kováts indexes) with those of reference substances and by comparison with the NIST mass spectral search software v2.0 (<http://chemdata.nist.gov/mass-spc/ms-search/>) using the libraries NIST 98 library and Adams (2001) library. Different concentrations of several pure standards were used to create an external standard curve used for SPME quantitative measurements.

RNA extraction from Arabidopsis WT and *pdko3* leaves upon HW and MD

After each experiment, leaves were collected and immediately frozen in liquid nitrogen. One hundred milligram of frozen HW and MD leaves were ground in liquid nitrogen with mortar and pestle. Total RNA was isolated using Qiagen RNeasy Plant RNA kit and RNase-Free DNase set (Qiagen, <http://www.qiagen.com/>). Sample quality and quantity was checked by using the RNA 6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies) according to manufacturer's instructions. Quantification of RNA was also confirmed spectrophotometrically by using a NanoDrop ND-1000 (Thermo Fisher Scientific, <http://www.thermofisher.com/>).

Quantitative real-time PCR (qPCR) reaction conditions and primers

First strand cDNA synthesis was accomplished with 2 µg of total RNA and random primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, <http://www.appliedbiosystems.com>), according to the manufacturer's recommendations. All experiments were performed on a Stratagene Mx3000P Real-Time System (Agilent) using SYBR green I with ROX as an internal loading standard. The reaction was performed with 25 µl of mixture consisting of 12.5 µl of 2× Maxima™ SYBR Green qPCR Master Mix (Fermentas International, Inc, <http://www.thermoscientificbio.com/fermentas/>), 0.5 µl of cDNA and 100 nm primers (Integrated DNA Technologies, <http://eu.idtdna.com>). Controls included non-RT controls (using total RNA without reverse transcription to monitor for genomic DNA contamination) and non-template controls (water template). Specifically, PCR conditions were the following: *AOS*, *OPR3*, *GPS1*, *FPS*, *TPS03*, *ACT1*, *GAPC2*, *UBP6*, *eEF1Balpha2*: 10 min at 95°C, 40 cycles of 15 sec at 95°C, 20 sec at 57°C, and 30 sec at 72°C; *TPS04*, *CYP82G1*, *TPS21*: 10 min at 95°C, 40 cycles of 15 sec at 95°C, 45 sec at 58°C, and 30 sec at 72°C. Fluorescence was read following each annealing and extension phase. All runs were followed by a melting curve analysis from 55 to 95°C. The linear range of template concentration to threshold cycle value (C_t value) was determined by performing a dilution series using cDNA from three independent RNA extractions analyzed in three technical replicates. All primers were designed using primer 3 software (Rozen and Skaletsky, 2000), except primers for *TPS03*, *TPS04*, *CYP82G1* already reported in literature (Snoeren *et al.*, 2010). Primer efficiencies for all primers pairs were calculated using the standard curve method (Pfaffl, 2001). Four different reference genes cytoplasmic glyceraldehyde-3-phosphate dehydrogenase, (*GAPC2*), ubiquitin specific protease 6 (*UBP6*), Actin1 (*ACT1*) and the elongation factor 1B alpha-subunit 2 (*eEF1Balpha2*) were used to normalize the results of the real-time PCR. The best of the four genes was selected using the normfinder software (Andersen *et al.*, 2004); the most stable gene was the elongation factor 1B alpha-subunit 2. Primers used for real-time PCR are reported in Table S1.

All amplification plots were analyzed with the MX3000P™ software (Agilent) to obtain C_t values. Relative RNA levels were calibrated and normalized with the level of the elongation factor 1B alpha-subunit 2 mRNA.

Statistical analyses

For V_m measurements, the obtained data were treated by using the stem-and-leaf function of systat 10 (<http://www.systat.com/>) in order to calculate the lower and upper hinge from the Gaussian distribution of values. The data were then filtered and the mean value was calculated along with the SE. Paired *t*-test and Bonferroni adjusted probability were used to assess the difference between treatments and the control. For all other experiments, at least five samples per treatment group were used for the statistical analysis of the data. Data are expressed as mean values ± standard error. To compare the control and the treatment groups, analysis of variance (anova) and Tukey's test were performed.

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Ancillary

Supporting Information

Figure S1. Feeding experiments with *Arabidopsis* WT and *pdko3* on *Spodoptera littoralis*. Third-fourth instar *S. littoralis* larvae were allowed to feed on *Arabidopsis* WT and *pdko3* fully expanded leaves for several days. No significant differences were observed in growth weight between larvae feeding on WT and *pdko3* plants.

Table S1. Primers used for real-time PCR. The length of PCR products ranged from 103 to 245 bp.

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