Functional Convergence of Oxylipin and Abscisic Acid Pathways Controls Stomatal Closure in Response to Drought[1][W][OPEN]

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Membranes are primary sites of perception of environmental stimuli. Polyunsaturated fatty acids are major structural constituents of membranes that also function as modulators of a multitude of signal transduction pathways evoked by environmental stimuli. Different stresses induce production of a distinct blend of oxygenated polyunsaturated fatty acids, "oxylipins." We employed three Arabidopsis (*Arabidopsis thaliana*) ecotypes to examine the oxylipin signature in response to specific stresses and determined that wounding and drought differentially alter oxylipin profiles, particularly the allene oxide synthase branch of the oxylipin pathway, responsible for production of jasmonic acid (JA) and its precursor 12-oxo-phytodienoic acid (12-OPDA). Specifically, wounding induced both 12-OPDA and JA levels, whereas drought induced only the precursor 12-OPDA. Levels of the classical stress phytohormone abscisic acid (ABA) were also mainly enhanced by drought and little by wounding. To explore the role of 12-OPDA in plant drought responses, we generated a range of transgenic lines and exploited the existing mutant plants that differ in their levels of stress-inducible 12-OPDA but display similar ABA levels. The plants producing higher 12-OPDA levels exhibited enhanced drought tolerance and reduced stomatal aperture. Furthermore, exogenously applied ABA and 12-OPDA, individually or combined, promote stomatal closure of ABA and allene oxide synthase biosynthetic mutants, albeit most effectively when combined. Using tomato (*Solanum lycopersicum*) and *Brassica napus* verified the potency of this combination in inducing stomatal closure in plants other than Arabidopsis. These data have identified drought as a stress signal that uncouples the conversion of 12-OPDA to JA and have revealed 12-OPDA as a drought-responsive regulator of stomatal closure functioning most effectively together with ABA.

To colonize a diverse range of environments successfully, plants have developed converging functional pathways to synthesize an array of secondary metabolites for their protection against hostile conditions. For example, in response to environmental challenges, the oxylipin pathway induces the de novo synthesis of biologically active compounds called "oxylipins," derivatives of oxygenated polyunsaturated fatty acids (Feussner and Wasternack, 2002; Howe and Schilmiller, 2002). Among the oxylipin pathways, the enzymes allene oxide synthase (AOS) and hydroperoxide lyase (HPL) are considered to partition two major branches that compete for the same substrates and are critical plant stress response pathways (Chehab et al., 2008).

Production of the AOS pathway metabolites 12-oxo-phytodienoic acid (12-OPDA) and jasmonic acid (JA) originates from α-linolenic acid of chloroplast membranes (Feussner and Wasternack, 2002). Oxygenation of α-linolenic acid by a 13-lipoxygenase followed by the action of AOS forms an unstable allene oxide that is subsequently cyclized by an allene oxide cyclase to form 12-OPDA (Stenzel et al., 2012). 12-OPDA is the end product of the plastid-localized part of the pathway (Stintzi and Browse, 2000; Schaller and Stintzi, 2009). 12-OPDA is then translocated to the peroxisome where it is reduced by 12-OPDA reductase3 (OPR3) and subsequently activated by CoA ester prior to undergoing three rounds of β-oxidation to form JA (Schaller et al., 2000; Koo et al., 2006; Kienow et al., 2008). 12-OPDA is also a signaling molecule with both overlapping and distinct functions from JA. The Arabidopsis (*Arabidopsis thaliana*) *opr3* mutant is deficient in JA synthesis but accumulates 12-OPDA and displays wild-type resistance to the dipteran *Bradysia impatiens* and to the fungal pathogen *Alternaria brassicicola*,

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generally considered JA-dependent responses (Stintzi et al., 2001). In addition, expression studies have identified genes induced by 12-OPDA but not by JA or methyl jasmonate (MeJA; Kramell et al., 2000; Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008). These studies collectively show that 12-OPDA mediates gene expression with or without the canonical JA signaling framework (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008).

The HPL branch of the oxylipin pathway produces aldehydes and corresponding alcohols. The first enzyme in the pathway is encoded by one or more *HPL* genes, differing in their subcellular localization, including microsomes (Pérez et al., 1999), lipid bodies (Mita et al., 2005), and the outer envelope of chloroplasts (Froehlich et al., 2001), and in some cases, with no specific localization in a particular organelle (Noordermeer et al., 2000). This variation in the number of genes and subcellular localization of their encoded enzymes is suggestive of the differential regulation of this pathway and, ultimately, the diversity of their responses, potentially tailored to the nature of stimuli.

We have previously identified three rice (*Oryza sativa*) HPLs (HPL1 through HPL3) differing in their enzyme kinetics and substrate preference. Expression of these enzymes in Arabidopsis accession Columbia (Col-0), a natural *hpl* loss-of-function mutant, reestablished the production of the pathway metabolites (Chehab et al., 2006) and revealed the key role of HPL-derived metabolites in plant stress signaling (Chehab et al., 2008).

The HPL and AOS branches of the oxylipin pathway do not function independently; the signaling crosstalk between them is key to fine tuning plant adaptive responses to a diverse range of perturbations (Halitschke et al., 2004; Liu et al., 2012; Scala et al., 2013).

To gain deeper insight into the role of AOS- and HPL-derived metabolites in fine-tuning plant stress responses, we have (1) characterized the corresponding oxylipin signatures in response to wounding and drought in three Arabidopsis ecotypes, (2) generated a range of transgenic lines that produce varying blends of oxylipins tailored to the nature of the stress, (3) elucidated a JA-independent role for 12-OPDA in enhanced drought tolerance in part via regulation of stomatal aperture, and (4) reexamined the 12-OPDA-mediated regulation of stomatal aperture, alone or in combination with abscisic acid (ABA) in the model system Arabidopsis as well as in two crop species, namely tomato (*Solanum lycopersicum*) and *Brassica napus*. Unexpectedly, these analyses have identified drought as a stress signal that uncouples the conversion of 12-OPDA to JA and have revealed that 12-OPDA is a previously unrecognized regulator of stomatal closure in response to drought. This function of 12-OPDA, however, is most effective when combined with ABA, a phytohormone known to be essential for plant-adaptive responses to drought stress (Seki et al., 2007).

RESULTS

Wounding and Drought Induce Distinct Blends of Oxylipins

We determined oxylipin signatures of three Arabidopsis ecotypes (Wassilewskija [Ws], Landsberg *erecta* [L*er*], and Col-0) in response to wounding and drought stress. We selected Col-0, a natural loss-of-function mutant in *hpl* (Duan et al., 2005), together with the other two ecotypes that produce HPL- and AOS-derived metabolites. As control, we expanded the oxylipin profiling and measured the levels of the classical drought-responsive hormone ABA (Wasilewska et al., 2008; Kim et al., 2010).

These analyses display a stress-tailored oxylipin signature. As expected, the HPL-derived metabolites were undetectable in Col-0, with the exception of the insignificant levels of 3-hexenol produced nonenzymatically in wounded plants (Fig. 1, A and B). The levels of 3-hexenal in the other two ecotypes, L*er* and Ws, were induced in response to drought but remained at basal levels in wounded tissue (Fig. 1A). This drought-induced accumulation of 3-hexenal, the direct product of HPL enzyme, corroborates the earlier reports showing that drought elevates *HPL* transcript levels (Reymond et al., 2000; De Domenico et al., 2012). Basal levels of this metabolite in wounded tissue, however, are likely caused by its wound-induced conversion into emitted volatiles (Savchenko et al., 2013). By contrast, the levels of 3-hexenol were increased by wounding and decreased by drought stress compared with the control (Fig. 1B). This stress-tailored differential response is also apparent in all three ecotypes for the AOS-derived metabolites. Specifically, drought induced production of 12-OPDA in L*er* and Ws, whereas, in Col-0, these levels were equally induced by drought and wounding, potentially due to a higher available pool of substrates in Col-0, the ecotype with the dysfunctional *hpl* (Fig. 1C). Interestingly, however, JA levels in all the three ecotypes enhanced solely in response to wounding but remained at basal levels in all ecotypes exposed to 5 d of drought (Fig. 1D). This lack of drought-mediated induction of JA, despite copious levels of the biosynthetic precursor 12-OPDA, suggests that drought signaling has blocked the conversion of 12-OPDA to JA. In addition, we examined levels of these oxylipins in Col-0 ecotype at 12 and 36 h post drought treatment and show that while JA remained at the basal levels, 12-OPDA levels displayed an increasing trend with time post treatment (Supplemental Fig. S1, A and B).

Furthermore, similar to 12-OPDA, levels of the classical drought-responsive hormone ABA were also mainly enhanced by drought, and little by wounding (Fig. 1E).

This drought induction of both 12-OPDA and ABA led to the questions of whether, similarly to ABA, 12-OPDA signaling also elicits plant-adaptive responses to drought stress, and, if so, dependently or independently from ABA.

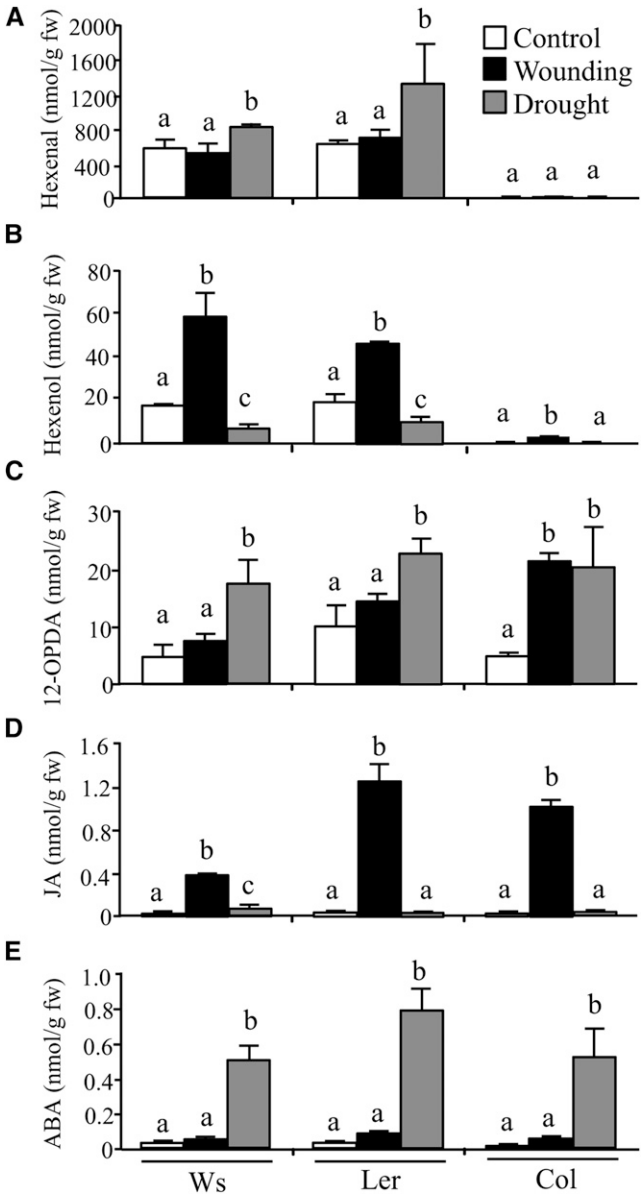

Figure 1. Wounding and drought induce distinct blends of oxylipins. A and B, HPL-derived metabolites cis-3-hexenal and cis-3-hexenol, C and D, AOS-derived metabolites 12-OPDA and JA, and E, ABA levels in three Arabidopsis ecotypes (Ws, Ler, and Col-0) examined in control (white bars), 90 min after wounding (black bars), and 5 d after withholding water (gray bars). Means ± SD of three independent biological replicates with three to four technical replicates are shown. Letters above bars indicate significant differences between stress treatments and the corresponding control in various ecotypes ($P < 0.05$).

Subcellular Localization of HPLs Influences the Stress-Inducible Levels of Jasmonates

To examine a potential role of various oxylipins in plant drought responses, we attempted to generate a series of transgenic lines with different HPL- and AOS-derived metabolites. However, our initial attempts deemed at manipulation of jasmonate branch via alteration of *AOS* expression levels led to silencing of the pathway and generation of sterile plants, a clear physiological response in plants unable to produce jasmonates (Stintzi and Browse, 2000; Park et al., 2002). By contrast, manipulation of HPL pathway metabolites through constitutive expression of rice *HPL2*- and *HPL3-GFP* fusion constructs driven by 35S promoter (designated as *HPL2* and *HPL3*) in Col-0 *hpl* mutant background led to production of HPL-derived metabolites. Two independent lines overexpressing equal levels of HPL enzymes, as determined by western-blot analyses, were employed for additional analyses (Figs. 2A; Supplemental Fig. S2A). High- resolution confocal microscopy established distinct subcellular localization of the two HPLs, that is, HPL2 is an extraplastidial enzyme, whereas HPL3 is plastid localized (Fig. 2B). The HPL2 extraplastidial localization is supported by previous results using chloroplast import assays but contradicts light microscopy localization studies identifying the enzyme as plastidial, potentially due to insufficient resolution of the earlier images (Chehab et al., 2006). This discrepancy prompted us to generate additional transgenic lines overexpressing extraplastidial HPL for comparison with *HPL2*. Thus, we removed the transit peptide from the HPL3-GFP fusion construct and produced additional transgenic lines, now designated *HPL3-TP*. The localization studies indicated that HPL3-TP is extraplastidial, concentrated in large structures that might be protein aggregates (Fig. 2B).

Next, the HPL-overexpressing transgenic lines were challenged with mechanical (wounding) and drought stress, and their respective oxylipins were analyzed. These data show restoration of 3-hexenal and 3-hexenol production in Col-0 background by all the constructs, albeit at varying levels (Fig. 2, C and D; Supplemental Fig. S2, B and C). The highest levels of 3-hexenal and 3-hexenol were in *HPL3* lines, as compared with the other transgenic lines. This is potentially due to a higher pool of available substrate in plastids and to the superior kinetics of this enzyme (Chehab et al., 2006). Importantly, however, the presence of HPL-derived metabolites in *HPL2* and *HPL3-TP* transgenic plants not only confirmed functionality of these extraplastidial enzymes, but also verified extraplastidial availability of the enzyme substrate (Fig. 2, C and D; Supplemental Fig. S2, B and C).

Profiling JA and 12-OPDA levels in control and stressed transgenic lines unexpectedly established that the subcellular localization of HPL enzyme alters the stress-inducible levels of jasmonates (Fig. 2, E and F; Supplemental Fig. S2, D and E). In particular, lines overexpressing plastidial HPL3 displayed the lowest induction in JA and 12-OPDA levels, as compared with lines expressing extraplastidial enzymes, in response to both stresses. This suggests that overexpression of plastidial HPL limits the substrate pool available to the AOS pathway. These data are supported by previous reports validating the competition between the HPL and AOS branches of oxylipin

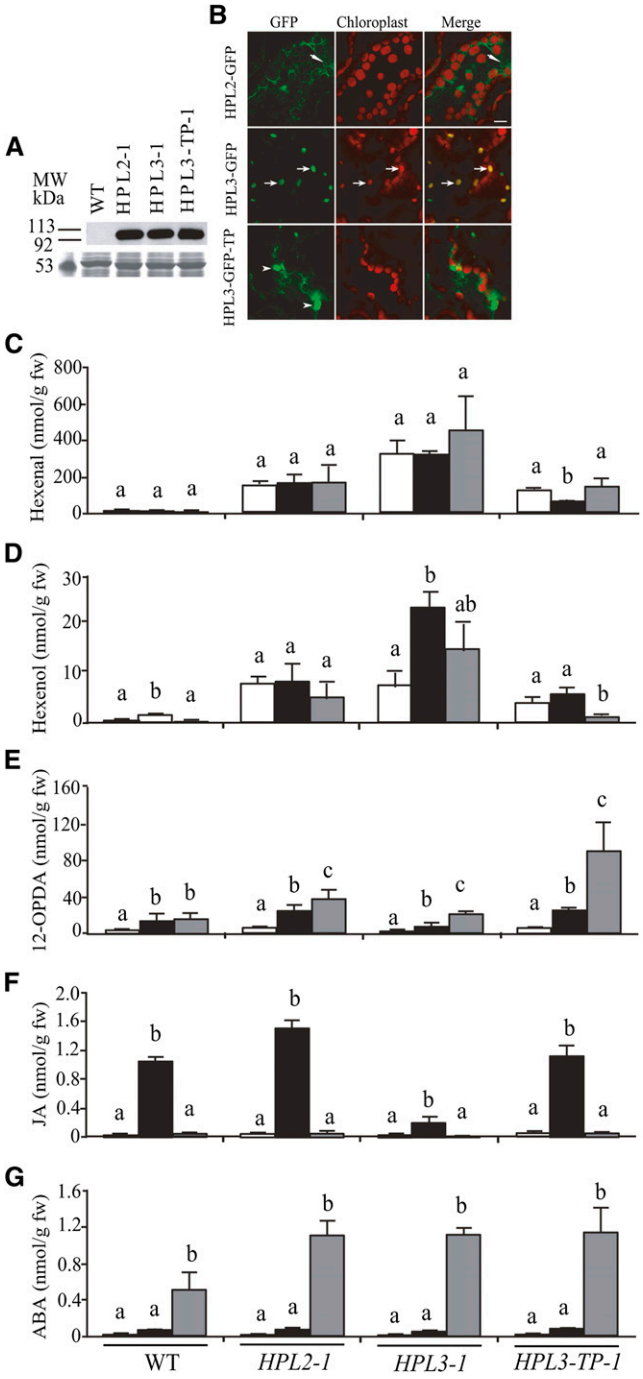

Figure 2. Subcellular localization of HPLs influences the stress-inducible levels of jasmonates. A, Western-blot analyses of transgenic lines expressing HPL-GFP fusion constructs for the two extraplastidial (HPL2 and HPL3-TP) enzymes and one plastidial HPL (HPL3) enzyme using anti-GFP antibody. Bottom section is the Coomassie blue staining of large Rubisco subunit displaying equal protein loading. B, Confocal images of Arabidopsis cotyledon cells at the epidermis and the mesophyll interface showing the subcellular distribution of the HPL-GFP fusions (pseudocolored green) and chlorophyll autofluorescence (pseudocolored red). Most of the HLP3-GFP was clearly restricted to chloroplasts in the epidermal layer, while the signal of the HLP2-GFP was found outside these organelles (arrows). The HPL3-GFP lacking the targeting peptide (HPL3-GFP-TP) was found outside the chloroplasts and concentrated in large structures that might be protein aggregates (arrows). Bar = 10 μm. C and D, Levels of HPL-derived metabolites cis-3-hexenal and cis-3-hexenol, E and F, AOS-derived metabolites 12-OPDA and JA, and G, ABA in untreated control (white bars), 90 min after wounding (black bars), and 5 d after withholding water (gray bars) in the wild type (WT) and HPL overexpressing transgenic lines. Means ± SD of three independent biological replicates with three technical replicates are shown. Letters above bars indicate significant differences between stress treatments and the corresponding control in various genotypes ($P < 0.05$).

pathway for the common substrate (Halitschke et al., 2004; Tong et al., 2012). In addition, these analyses further display induction of free 12-OPDA levels by both wounding and drought in all lines examined, most notably in drought-stressed plants overexpressing extraplastidial HPLs (Fig. 2E; Supplemental Fig. S2D). By contrast, JA levels were only induced by wounding but remained at basal levels in response to drought treatment, in spite of notable levels of the metabolic precursor 12-OPDA (Fig. 2, E and F; Supplemental Fig. S2).

The unexpected results displaying higher levels of stress-inducible 12-OPDA in overexpressing extraplastidial HPL lines could be rationalized by enhanced activation of AOS pathway by HPL-derived metabolites in conjunction with increased availability of plastidial substrate pool to the AOS branch in the absence of a functional plastidial HPL enzyme. Moreover, the lack of JA accumulation in drought-treated plants in spite of notable presence of the precursor, strongly suggest that JA production is not substrate limited, but rather its synthesis is uncoupled from 12-OPDA by a drought-specific signaling mechanism.

As control, we also examined ABA levels in these plants. These result show that drought also enhanced ABA levels in all plants examined and at similar levels in all transgenic lines (Fig. 2G; Supplemental Fig. S2F). This result is a clear indication of the suitability of these transgenic lines for studying a potential ABA-independent role of 12-OPDA in drought stress responses.

Drought Enhances 12-OPDA Level

To further examine the potential role of a JA-independent drought stress signaling in alteration of 12-OPDA levels, we exploited two mutant lines. One is an *aos* mutant unable to produce either 12-OPDA or JA. This mutant was initially generated in the trichomeless background (*glabrous1* [*gl1*], accession Col-0; Park et al., 2002), but we successfully segregated out the *gl-1* locus. The second is an *opr3* mutant in a Ws background capable of producing 12-OPDA but not JA (Stintzi and Browse, 2000). Jasmonate profiling of these mutant lines and their respective control backgrounds confirmed expected JA and 12-OPDA levels (Fig. 3, A and B). In agreement with metabolic profiles of *HPL*

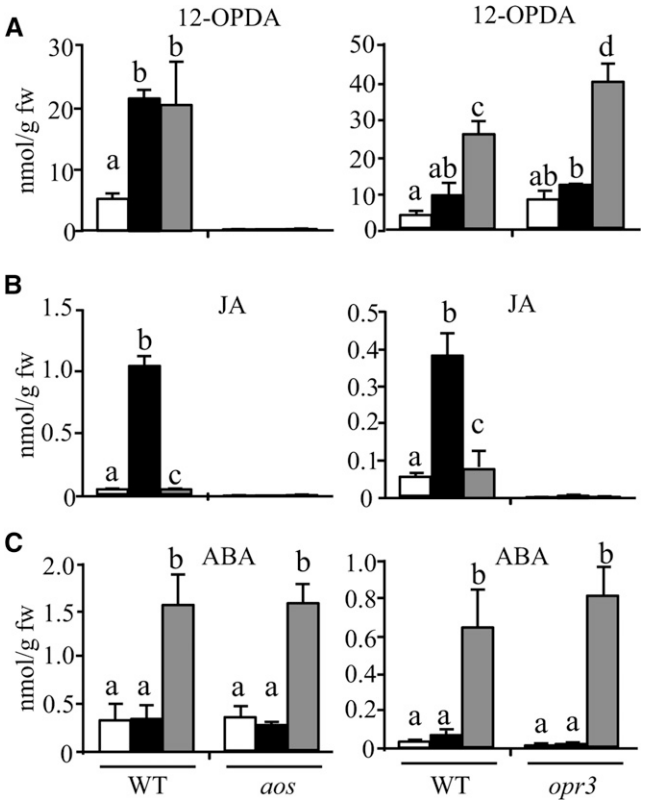

Figure 3. Drought enhances 12-OPDA level. Levels of 12-OPDA (A), JA (B), and ABA (C) in untreated *aos* and corresponding Col-0 background and *opr3* and corresponding Ws background (white bars) 90 min after wounding (black bars) and 5 d after withholding water (gray bars). Means ± SD of three independent biological replicates with three technical replicates are shown. Letters above bars indicate significant differences between stress treatments and the corresponding control in various genotypes ($P < 0.05$).

overexpression lines (Fig. 2, E and F), JA levels were induced by wounding but suppressed by drought in the wild-type backgrounds, whereas 12-OPDA levels were elevated by both stresses in wild-type ecotypes as well as in *opr3* mutant lines (Fig. 3, A and B). Importantly, drought induced ABA at almost equal levels in all these lines, indicating that this induction is independent of jasmonates (Fig. 3C).

Accumulation of 12-OPDA Reduces Stomatal Aperture and Enhances Drought Tolerance

To determine the correlation between drought-induced levels of 12-OPDA and physiological responses, we examined the survival rate of all the transgenic and mutant plants described above. These data clearly display a strong correlation between survival rate and 12-OPDA level in drought-stressed plants (Fig. 4, A, C, and E; Supplemental Fig. S3, A and B). Specifically, *HPL3-TP* and *opr3*, plants with highest 12-OPDA levels (Fig. 2E; Supplemental Figs. S2D and S3A), displayed greatest survival rate, while *aos* mutant lines unable to produce 12-OPDA exhibited lowest survival rate in response to drought (Fig. 4, A–F). In addition, we observed an inverse correlation between 12-OPDA level and stomatal aperture

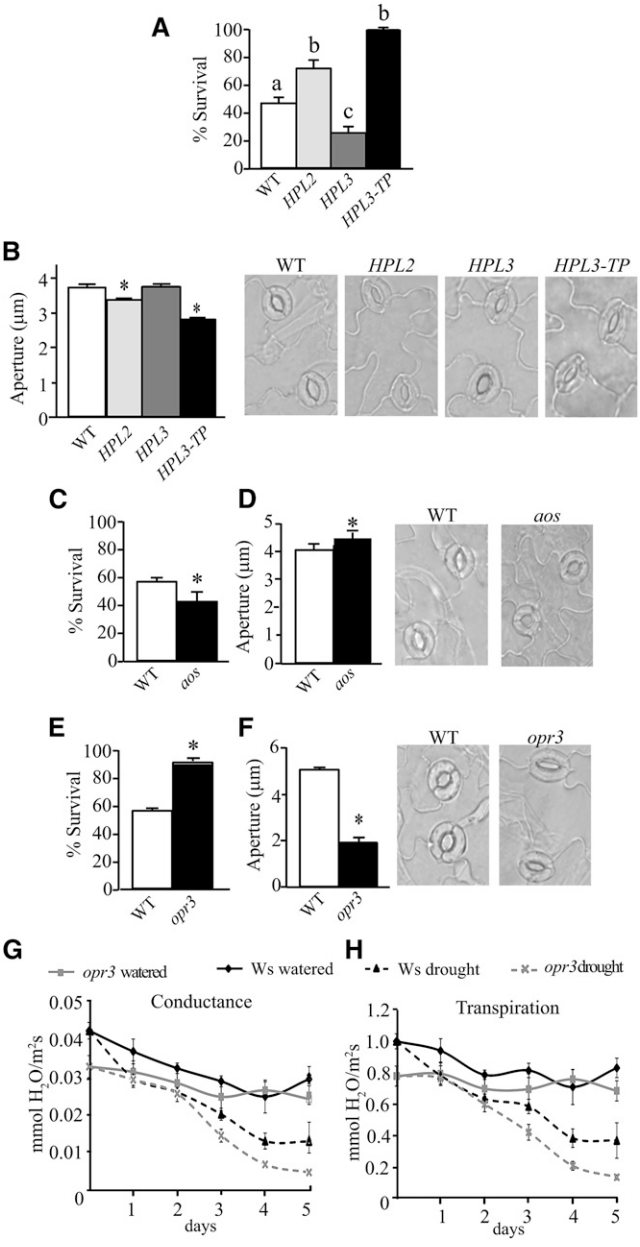

Figure 4. Accumulation of 12-OPDA reduces stomatal aperture and enhances drought tolerance. A, C, and E, Survival rate measured in the wild type (WT), *HPL* overexpressors, and *aos* and *opr3* mutant lines and their corresponding wild-type genotypes exposed to 10 d of drought followed by 7 d of watering. B, D, and F, Stomatal apertures measured in epidermal peels of the wild type, *HPL* overexpressors, and *aos* and *opr3* mutants and their corresponding wild-type genotypes. Representative stomatal images are shown. G and H, Stomatal conductance and transpiration rate in well-watered and drought-stressed *opr3* mutant and the corresponding wild-type genotype Ws. Means ± SE of six to nine independent biological replicates are shown. Asterisk denotes significant difference ($P < 0.0001$).

(Figs. 3A and 4F; Supplemental Fig. S3C). We further verified these data by examining the stomatal conductance and transpiration rates of watered and dehydrated Ws and *opr3* plants over 5 d (Fig. 4, G and H). This result show the *opr3* mutant has lower stomatal conductance and, by extension, transpiration rate than that of the wild type, in spite of having comparable ABA levels. Collectively, these analyses suggest that in addition to ABA, the phytohormone 12-OPDA is also a regulator of stomatal aperture in response to drought.

External Application of 12-OPDA Promotes Stomatal Closure

To test if individual oxylipins promote stomatal closure, we analyzed the concentration-dependent effect of HPL- and AOS-derived metabolites on stomatal aperture in epidermal peels of Col-0. Among the HPL-derived metabolites examined, only high concentrations (50–100 μM) of 3-hexenal slightly reduced the stomatal aperture (Fig. 5A; Supplemental Fig. S4A).

Among the AOS-derived metabolites, application of MeJA reduces stomatal aperture nonlinearly and most effectively at high concentrations of up to 200 μM (Fig. 5B). This result is in disagreement with previous findings showing complete or almost complete stomatal closure in epidermal peels of *Commelina benghalensis* and *Nicotiana glauca* treated with either MeJA or ABA (Raghavendra and Reddy, 1987; Suhita et al., 2003). Our data are also in conflict with the report demonstrating that 10 μM of either MeJA or ABA equally and effectively reduce the stomata aperture in L*er* (Suhita et al., 2004). By contrast, our findings consistently show that under no circumstances are equal concentrations of ABA and MeJA similarly potent in stomatal closure. The ability of MeJA to induce stomatal closure remains controversial, because in support of our data, a recent report showed that MeJA at concentrations up to 100 μM is ineffective in reducing stomatal aperture (Montillet et al., 2013). Furthermore, dose response curve studies show that maximum reduction of stomatal aperture is achieved by 12-OPDA at approximately 5-fold-lower concentrations than that of MeJA (Fig. 5B).

Because of enhanced levels of both hexenal and 12-OPDA in drought-stressed plants (Figs. 1, A and C, and 2, C and E; Supplemental Fig. S2, B and D), we questioned whether 12-OPDA and 3-hexenal could cooperatively reduce stomatal aperture. These data show that coapplication of 3-hexenal (100 μM) together with 12-OPDA reduced the aperture by an additional approximately 20% as compared with 12-OPDA alone (Supplemental Fig. S4B) for 12-OPDA concentrations up to 2 μM.

We next studied the dose response effect of ABA on stomatal closure, alone or with various concentrations of 12-OPDA (Fig. 5C; Supplemental Fig. S4B). These data illustrate that ABA is the most effective phytohormone in reducing the stomatal aperture (Fig. 5, A–C). However, stomatal closure is most apparent when ABA and 12-OPDA are coapplied (Fig. 5C). To avoid potential nonspecific inhibitory effects of 12-OPDA caused by its reactive α,β-unsaturated carbonyl structure (Alméras et al., 2003; Mueller et al., 2008), the coapplication experiments were performed with low concentrations (2 μM) of 12-OPDA.

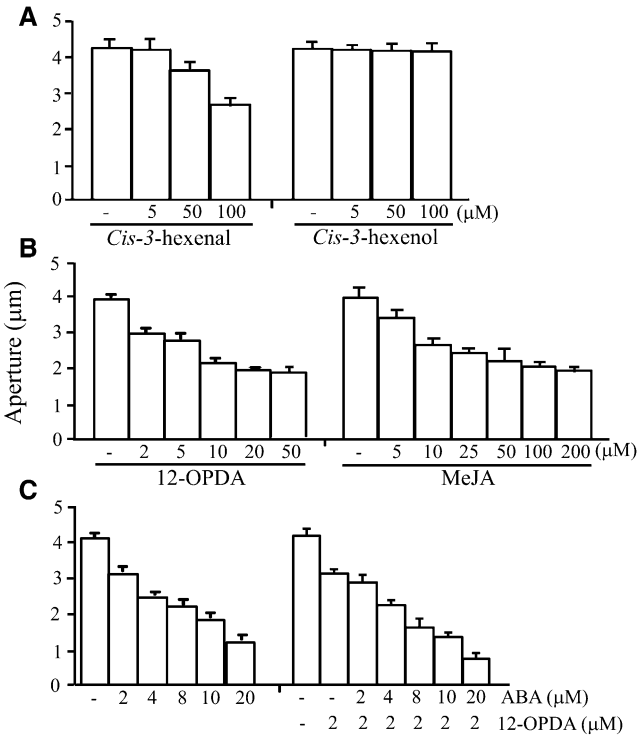

Figure 5. External application of 12-OPDA promotes stomatal closure. Measurements of stomatal aperture in epidermal peels of Col-0 after application of various concentrations of cis-*3*-hexenal and cis-*3*-hexenol (A), 12-OPDA and MeJA (B), and ABA alone or together with 12-OPDA (C). Data represent the means of minimum 90 measurements ± SE.

12-OPDA Regulates Stomatal Aperture Independently and Cooperatively with ABA

To examine the regulatory role of 12-OPDA and ABA, we next exploited stomatal aperture behavior in *aba2-1*, blocked in the conversion of xanthoxin to ABA aldehyde (Léon-Kloosterziel et al., 1996; Schwartz et al., 1997), and *aos* mutant lines. Application of 12-OPDA reduces the stomatal aperture in epidermal peels of *aos* line, albeit more effectively than in *aba2-1* line (Fig. 6, A and B), supporting the notion of a JA-independent function of 12-OPDA and the dominant role of ABA. Importantly, although stomatal apertures in both wild-type and *aba2-1* lines are equally reduced by ABA, this reduction is most prominent in the presence of both 12-OPDA and ABA, suggesting the importance of the cooperative signaling function of these two phytohormones (Fig. 6B).

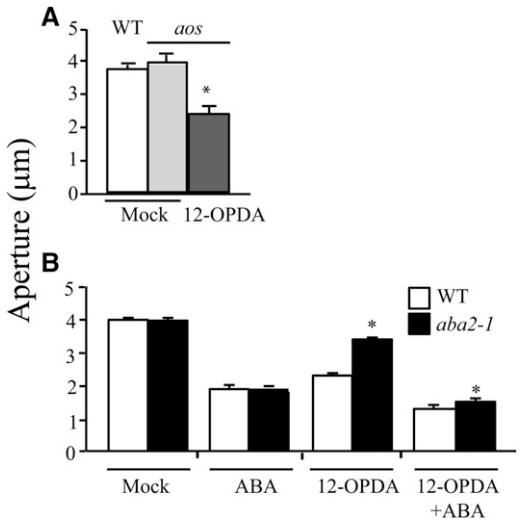

Figure 6. 12-OPDA regulates stomatal aperture independently and cooperatively with ABA. Stomatal apertures measurements of the wild type (WT) and *aos* mutant lines in the presence and absence of 2 μM 12-OPDA (A), the wild type and *aba2-1* mutant in the presence of 2 μM 12-OPDA and 10 μM ABA, individually or combined (B). Means ± SD of 90 independent measurements are shown. Asterisk denotes significant difference in aperture under a given treatment ($P < 0.0001$).

Cooperative Function of 12-OPDA and ABA Is Not Limited to Arabidopsis

To determine whether coapplication of 12-OPDA and ABA would modulate the stomatal aperture in plants other than Arabidopsis, we prepared tomato and *B. napus* epidermal strips and treated each with these metabolites. Subsequent measurements of stomatal aperture in both plants showed that application of either ABA or 12-OPDA promote stomatal closure, albeit most effectively when combined (Fig. 7, A and B). These data provide compelling evidence for a cooperative signaling function of 12-OPDA and ABA in plants other than Arabidopsis.

DISCUSSION

Drought stress is known to alter the balance of several plant hormones and modify their interactions, which ultimately can alter stomatal function (Acharya and Assmann, 2009). Among the phytohormones, ABA plays the most important role, limiting stomatal apertures under water stress conditions (Pospisilova, 2003; Kim et al., 2010). The lipid-derived hormone JA is also implicated as a key player based on its accumulation during drought and its positive regulatory role in stomatal closure (Creelman and Mullet, 1995; Suhita et al., 2004; Munemasa et al., 2007). Interestingly, however, JA accumulation in drought is not stable; water-stressed soybeans (*Glycine max*) exhibit a transient increase in JA levels, followed by a decrease to below wild-type levels by 4 h (Creelman and Mullet, 1995). Our data confirms that 5 d after drought stress, JA remains at basal levels, while 12-OPDA levels increase. These findings suggest that certain stresses, such as drought, either block translocation of 12-OPDA and/or inhibit the subsequent peroxisomal steps of JA biosynthesis. Furthermore, these data suggest that in contrast to JA, 12-OPDA-dependent signaling cascade is devoted to best cope with certain long-lasting stresses. The role of 12-OPDA in drought resistance is further supported by a recent study showing the importance of 13-lipoxygenases such as LOX6, an enzyme required for production of basal 12-OPDA levels in leaves and roots in plant drought resistance (Grebner et al., 2013).

Moreover, we illustrate that 12-OPDA regulates stomatal aperture in an ABA-deficient mutant *aba2-1*, albeit less effectively than ABA. By contrast, studies conducted on ABA biosynthetic mutant *sitiens* tomato showed that JA application led to stomatal closure only when petioles were preincubated with ABA (Herde et al., 1997). Similarly, studies on ABA-deficient Arabidopsis mutant *aba2-2* or in plants treated by fluridone, an inhibitor of ABA biosynthesis, demonstrated that MeJA was no longer able to promote stomatal closure (Hossain et al., 2011). In fact, an additional microarray study suggests that MeJA reduced expression of ABA-responsive stress-related genes (Jung et al., 2007). Collectively, these data suggest that 12-OPDA is functional in potentiating stomatal movement, whereas JA is active only when ABA endogenous levels reach a critical threshold. An ABA-independent function of

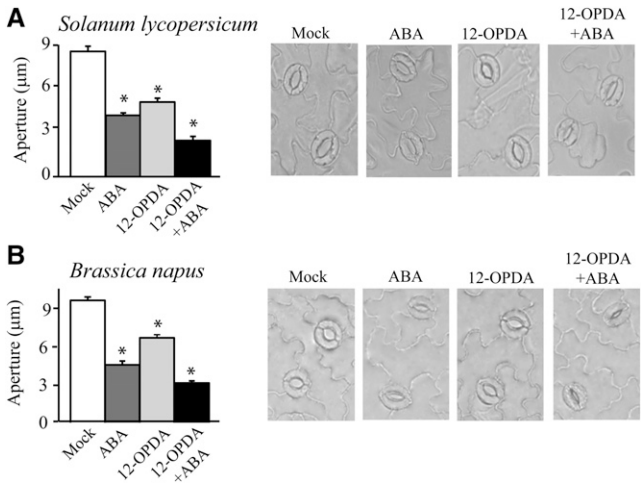

Figure 7. Cooperative function of 12-OPDA and ABA is not limited to Arabidopsis. Stomatal aperture measurements in epidermal strips of tomato (A) and *B. napus* (B) in the presence of 10 μM ABA or 2 μM 12-OPDA, individually or combined. Representative stomatal images are shown. Means ± SD of 90 independent measurements are shown. Asterisk denotes significant difference in aperture under a given treatment and the corresponding mock treatment ($P < 0.0001$).

12-OPDA in reducing stomata aperture is also reported as a functional innate immunity mechanism to ward plants against pathogens (Montillet et al., 2013).

Stress-specific distinct roles of JA and 12-OPDA in regulation of gene expression are further exemplified by stress-induced expression of *AtPHO1;H10*, a member of the Arabidopsis *PHO1* gene family (Ribot et al., 2008). Induction of *ATPHO1;H10* expression by wounding mainly involves the OPDA-COI1 (for Coronatine Insensitive1) pathway and is independent of the ABA pathway, whereas, following dehydration, both the ABA and OPDA-COI1 pathway are needed for maximal *AtPHO1;H10* expression. Interestingly, however, it is reported that under moderate drought treatment, while ABA biosynthetic and signaling mutants displayed sensitivity, coi1 and jasmonate-insensitive1 mutant plants were found to be significantly resistant as compared with the wild type (Harb et al., 2010). These authors propose a three-stage (early, intermediate, and late) drought response model and further show that an early stage drought leads to accumulation of high ABA levels in concomitant with induction of ABA biosynthesis and signaling genes but without significant differential expression of JA pathway genes, thus suggesting that endogenous JA levels in combination with high ABA is sufficient to stimulate the initial responses necessary for drought acclimation. They further propose that high JA levels under drought stress might negatively affect plant growth, specifically cellulose synthesis, as a key determinant in plant resistance to fungal pathogens. Ample additional evidence further supports the functional convergence of ABA and jasmonate signaling pathways in plant-pathogen interactions, where ABA can either play a synergistic or antagonistic role with jasmonates depending on the pathogen (Anderson et al., 2004; Mauch-Mani and Mauch, 2005; Adie et al., 2007).

The cooperative function of 12-OPDA and ABA is not limited to plant responses to environmental cues but expands to developmental processes such as regulation of seed germination in Arabidopsis. A combination of genetics and feeding experiments has demonstrated that 12-OPDA interacts with ABA, inhibits seed germination, and increases ABA INSENSITIVE5 protein (Dave et al., 2011; Dave and Graham, 2012). These results, collectively, further highlight the importance of fine-tuning of jasmonate signatures in response to environmental and developmental signals and signify the stimulus-dependent balance in levels of plant hormones and, by extension, their interactions.

Here, we have identified drought as a stress signal that alters jasmonate signature by blocking the conversion of 12-OPDA to JA and further uncovered 12-OPDA as a functional convergence point of oxylipin and ABA pathways to control stomatal aperture in plant-adaptive responses to drought stress. Further investigations will be necessary to establish how drought signaling blocks JA biosynthesis and how 12-OPDA in particular acts together with ABA to elicit stomatal closure in response to drought stress.

MATERIALS AND METHODS

Generation of Transgenic Lines and Plant Growth

Generation of transgenic plant overexpressing *HPL2* and *HPL3* is as described previously (Chehab et al., 2006). To generate *HPL3-TP* lines, 45-bp nucleotides were removed from the amino terminus of *HPL3* complementary DNA, followed by cloning of the remainder fragment as a GFP fusion into pB7WGF2 vector. Plants were grown in soil in a 16-h-light/8-h-dark cycle at 22°C. Three-week-old plants were used for assays if not stated otherwise.

Western-Blot Analysis

Total plant proteins were extracted from Arabidopsis (*Arabidopsis thaliana*) leaves by grinding tissue in SDS sample buffer. Proteins (5 μg) were separated by SDS-PAGE (12% [w/v] gels) and transferred to nitrocellulose membrane. Clontech Living Colors GFP Monoclonal Antibody was used as primary antibody and goat anti-mouse HRP conjugate (Pierce) as secondary antibodies to detect HPL-GFP fusion proteins in a standard western-blot procedure.

Confocal Microscopy

Confocal images of Arabidopsis cotyledon cells at the epidermis and the mesophyll interface showing the subcellular distribution of the HPL-GFP fusions (pseudocolored green) and chlorophyll autofluorescence (pseudocolored red, Fig. 2). Most of the HLP3-GFP was clearly restricted to chloroplasts, while the signal of the HLP2-GFP was found outside these organelles. The HPL3-GFP lacking the targeting peptide (HPL3-GFP-TP) was found outside the chloroplasts and concentrated in large structures that might be protein aggregates (arrowheads). Bar = 10 μm.

An inverted laser scanning confocal microscope (LSM510 META, Carl Zeiss) and a 63×/1.40 oil immersion objective were used for confocal analyses. Imaging of cells expressing the GFP fusions was carried out using 488-nm excitation of an argon laser line for GFP and 543-nm He/Ne for chlorophyll autofluorescence; a 488/543 beam splitter and BP505-530 and LP560 emission filters were used for acquisition of the GFP and chlorophyll autofluorescence signal, respectively.

Extraction and Quantification of Phytohormones

Extraction of JA, 12-OPDA, ABA, and HPL-derived metabolites was carried out as previously described (Schmelz et al., 2003; Chehab et al., 2008).

Stress Treatment and Survival Test

Drought experiments were performed on individual plants grown in pots containing the same amount of soil and watered equally for 3 weeks before watering was withheld for 10 d. Subsequently, plants were extensively watered, and their survival rate was scored 7 d later. Metabolite analyses, however, were performed on plants exposed to 5 d of drought soon after the appearance of the first signs of wilting.

Mechanical wounding was performed as previously described (Walley et al., 2007). Harvested tissues were immediately frozen in liquid nitrogen and stored at –80°C prior to analysis.

Genotyping

All plants were genotyped using primers shown in Supplemental Table S1.

Stomatal Aperture Measurements

Abaxial (lower) epidermal strips of Arabidopsis, tomato (*Solanum lycopersicum*), and *Brassica napus* were adhered to glass cover slips using Telesis V adhesive (Premiere Products) and subsequently treated as previously described (Kolla et al., 2007). The epidermal strips incubated with various phytohormones were irradiated with white light for 2 h. The width of the stomatal aperture was measured by light microscope (Nikon OPTIPHOT 2) using a precalibrated ocular micrometer. Each stomatal aperture value is based on an average of at least 90 measurements from 30 randomly monitored apertures in epidermal strips. Images of the stomata were captured by the monochrome camera (Q imaging Retiga EXi).

Gas Exchange Measurements

These measurements were performed with a Li-6400 photosynthesis system (Li-Cor Biosciences), using 10 plants per treatment per genotype, daily for 5 d starting on the first day of drought treatment. Cuvette conditions were: photosynthetic photon flux density of 150 μmol m^{-2} s^{-1}, flow rate of 100 μmol s^{-1}, reference CO_2 of 400 μmol mol^{-1}, and chamber temperature of 22°C.

Statistical Analyses

To determine statistical significance of stress treatments in various genotypes, we performed one-way ANOVAs with SPSS statistics software. The difference was considered significant if $P < 0.05$.

Supplemental Data

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

Supplemental Figure S1. Profiles of JA and 12-OPDA levels post drought stress.

Supplemental Figure S2. Drought stress signaling uncouples conversion of 12-OPDA to JA.

Supplemental Figure S3. Plants overexpressing extra plastidial HLP are more drought resistance than the wild-type control.

Supplemental Figure S4. External application of 12-OPDA promotes stomatal closure.

Supplemental Table S1. List of primer sequences.

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