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Waterlogging tolerance rendered by oxylipin-mediated metabolic reprogramming in *Arabidopsis*

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Abstract

Environmental stresses induce production of oxylipins synthesized by the two main biosynthetic branches, allene oxide synthase (AOS) and hydroperoxide lyase (HPL). Here, we investigate how waterlogging-mediated alteration of AOS- and HPL-derived metabolic profile results in modulation of central metabolism and ultimately enhanced tolerance to this environmental stress in *Arabidopsis thaliana*. Waterlogging leads to increased levels of AOS- and HPL-derived metabolites, and studies of genotypes lacking either one or both branches further support the key function of these oxylipins in waterlogging tolerance. Targeted quantitative metabolic profiling revealed oxylipin-dependent alterations in selected primary metabolites, and glycolytic and citric acid cycle intermediates, as well as a prominent shift in sucrose cleavage, hexose activation, the methionine salvage pathway, shikimate pathway, antioxidant system, and energy metabolism in genotypes differing in the presence of one or both functional branches of the oxylipin biosynthesis pathway. Interestingly, despite some distinct metabolic alterations caused specifically by individual branches, overexpression of *HPL* partially or fully alleviates the majority of altered metabolic profiles observed in AOS-depleted lines. Collectively, these data identify the key role of AOS- and HPL-derived oxylipins in altering central metabolism, and further provide a metabolic platform targeted at identification of gene candidates for enhancing plant tolerance to waterlogging.

Keywords: Allene oxide synthase, central metabolism, hydroperoxide lyase, metabolite profiling, oxylipins, waterlogging stress.

Introduction

The periodic flooding/waterlogging across the globe impacts soil quality and reduces crop yield. During the most commonly occurring temporary waterlogging, water-saturated soil not only reduces oxygen availability to roots/rhizomes, but also alters the organic and inorganic composition of soil and accessibility of nutrients, changes the microbial environment, and leads to formation of toxic compounds, and ultimately reduced

crop yield. The impact of lowered oxygen levels on altering metabolism, hormonal status, and re-programming of the gene expression profile is well established (Vashisht *et al.*, 2011; van Dongen and Licausi, 2015; Voesenek and Bailey-Serres, 2015; Gasch *et al.*, 2016; van Veen *et al.*, 2016). Subsequent re-exposure of plant tissue to oxygen, a process known as ‘reoxygenation’, induces a severe oxidative stress. While roots’ responses

to waterlogging are well studied, the molecular nature of the adaptive processes occurring in aboveground tissues of waterlogged plants is not fully understood. As such, gaining an in-depth knowledge of a universal mechanism(s) underlying tolerance will be crucial for successful agricultural practices under these challenging circumstances.

Flooding and waterlogging can have profound effects on plant central metabolism, depending on the duration, the developmental stage, the organ, and whether the entire plant or just part of it (typically the root system) is submerged (Sasidharan et al., 2017). Submergence generally leads to reduced levels of oxygen, and simultaneously results in elevated levels of ethylene, nitric oxide, and free radicals (van Dongen and Licausi, 2015). These signals are accompanied by reduced energy and carbon availability, due to reduced photosynthesis, impaired aerobic respiration, and enhanced glycolysis in favor of fermentation. This altogether is translated into reductions in energy-demanding metabolic reactions (e.g. protein translation) and transport processes, which ultimately can cause growth retardation and/or death. The extent to which these alterations occur depends strongly on species' tolerance to oxygen deprivation that varies widely, as evidenced in natural variants of *Arabidopsis thaliana* (Vashisht et al., 2011). In spite of notable variations, some transcriptional core responses have been defined (Gasch et al., 2016; van Veen et al., 2016).

The lipid-derived signaling molecules oxylipins are known for their broad spectrum of regulatory functions and are implicated in regulation of plant adaptive responses under prevailing environmental conditions. Oxylipin biosynthesis is initiated in chloroplasts by lipases, which cleave fatty acids from the glycerol backbone, followed by oxygenation of free fatty acids by lipoxygenases, and thus formation of hydroperoxides of fatty acids. In *Arabidopsis*, allene oxide synthase (AOS) and 13-hydroperoxide lyase (HPL) are enzymes of the two characterized branches of the oxylipin biosynthesis pathway that compete for the common substrate—the hydroperoxide of linolenic acid [13-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid—13-HPOT] (Fig. 1A). 13-HPOT is converted into (9S,13S)-12-oxo-phytodienoic acid (12-OPDA) by the consequent action of AOS and allene oxide cyclase (Farmaki et al., 2007). Subsequent steps in jasmonic acid (JA) biosynthesis occur in peroxisomes, where reduction of 12-OPDA by 12-OPDA reductase (Stintzi and Browse, 2000), followed by three cycles of β -oxidation, results in JA production (Li et al., 2005). In the competing HPL branch, 13-HPL utilizes 13-HPOT as a substrate to form 12-carbon oxoacids and 6-carbon aldehydes, that can be further isomerized, reduced, and acetylated (Matsui et al., 2000). The functions of the AOS and HPL pathways in the regulation of plant responses to biotic challenges and abiotic stresses are well documented (Santino et al., 2013; Wasternack and Hause, 2013; Savchenko et al., 2014b; Kazan, 2015). Thus far, studies of alteration of plant stress responses by AOS pathway-derived metabolites have predominantly focused on the analyses of genes responsible for the synthesis of secondary metabolites (Gundlach et al., 1992; Brader et al., 2001; Zhao et al., 2005), but their regulatory function in central metabolism has remained an enigma (van der Fits and Memelink, 2000; Machado et al., 2013; Lu et al.,

2015; Machado et al., 2015). Some studies have demonstrated the role of jasmonates in the regulation of carbohydrates, as evinced by significant reduction of starch concentration in tobacco plants impaired in the jasmonate signaling pathway (Wang et al., 2014). Furthermore, in *Nicotiana attenuata*, JA regulates resistance to *Manduca sexta* through the control of sugar levels (Machado et al., 2015). It was even presumed that decreased herbivore weight gain during feeding on jasmonate-deficient plants is a result of the lower nutritional quality of these plants due to the absence of jasmonate-dependent regulation of primary metabolites (Machado et al., 2013; Lu et al., 2015). Exogenous jasmonate treatment has been shown to lead to altered starch concentration in the leaves of poplar (Babst et al., 2005), sugar levels in tulip stems (Skrzypek et al., 2005), and sugar and amino acid contents in tobacco and cabbage leaves (Hanik et al., 2010; Tytgat et al., 2013). In contrast to the AOS branch, the role of the HPL branch in regulation of primary metabolism and tolerance to waterlogging stress has not been studied before. We have shown previously that the activity of the HPL branch protects the photosynthetic apparatus from photoinhibition under excessive light through control of the levels of toxic fatty acid hydroperoxides and metabolites of the jasmonate pathway (Savchenko et al., 2017). On the other hand, HPL overexpression reduces plant drought tolerance, mainly negatively impacting the level of 12-OPDA, the metabolite with regulatory functions produced in the AOS branch (Savchenko et al., 2014a). These results raised the question of whether the HPL branch pathway functions indirectly through competition for fatty acid hydroperoxide substrate also used by the AOS pathway, or directly through regulatory function of the HPL branch-produced metabolites. In order to differentiate the biological functions of each oxylipin biosynthesis branch in the present study, we investigated the tolerance of genotypes lacking AOS and HPL branches of the oxylipin biosynthesis pathway individually and in all combinations to complex stress conditions such as waterlogging. Targeted metabolomics of these genotypes grown under normal or waterlogged conditions identified their role in altering central metabolism, and further provided a platform for identification of candidate gene(s) involved in modification of plant tolerance to waterlogging.

Materials and methods

Plants and growth conditions

Wild type (WT) *Arabidopsis thaliana* plants [Landsberg erecta (*Ler*) and Columbia-0 (*Col*)] and previously described transgenic lines with an altered oxylipin profile (Chehab et al., 2006, 2008; Savchenko et al., 2017) were employed (Table 1). Plants with an altered oxylipin profile were generated in WT *Col* plants and the trichomeless mutant *gl-1*. Mutation in *GL-1* (*GLABROUS-1*) coding for a MYB-like transcription factor essential for trichome initiation causes an almost glabrous trichome phenotype. WT *Col* plants and the trichomeless mutant *gl-1* (in the *Col* accession) (Park et al., 2002) are natural knockouts in the HPL gene (AT4G15440) due to a 10 nucleotide deletion in the first exon of the gene (Duan et al., 2005), but possess a functional AOS gene (AT5G42650), so these lines are designated in the text below as AOS *hpl GL-1* and AOS *hpl gl-1*, respectively. The *aos* knockout line in the trichomeless *gl-1* background (here designated as *aos hpl gl-1*) was previously purchased from the the *Arabidopsis* Biological Resource Center

Fig. 1. Waterlogging induces production of oxylipins. (A) Simplified scheme of the oxylipin biosynthesis pathway in Arabidopsis. Abbreviations: LOXs, lipoxygenases; AOS, allene oxide synthase; HPL, hydroperoxide lyase; 12-OPDA, 12-oxophytodienoic acid; OPR3, 12-OPDA reductase 3. (B–F) Levels of individual oxylipins in leaf tissues of different Arabidopsis genotypes. *Arabidopsis thaliana* genotypes [wild-type Landsberg *erecta* (*Ler*) ecotype with functional AOS and HPL branches, wild-type Columbia (*Col*) ecotype with a functional AOS branch and non-functional HPL branch, and *AOS HPL*, transgenic *Col* plants overexpressing rice *HPL3* with functional AOS and HPL branches] were grown for 3 weeks under normal conditions and then waterlogged for 5 d (gray bars) or continued to grow under normal conditions (white bars), before leaf tissue was collected for analyses. Means $\pm$ SD of three independent biological replicates comprised of bulked rosette leaves from eight plants are shown. Asterisks above the bars indicate a statistically significant difference ($P \leq 0.05$) between control and waterlogging-treated plants of the same genotype.

(Columbus, OH, USA; CS6149), and used for transformation with the *HPL3* gene from *Oryza sativa* as reported earlier (Chehab *et al.*, 2008) to generate the *aos HPL gl-1* line. Previously described plants overexpressing rice *HPL3* in the WT *Col* background (Chehab *et al.*, 2006) here are designated as *AOS HPL GL-1*. Later an *aos* loss-of-function mutant in the *GL-1* background (*aos hpl GL-1*) was generated by crossing WT *Col* with an *aos* loss-of-function mutant in the *gl-1* background, followed by generation of *aos HPL GL-1* by crossing *aos hpl GL-1* and *AOS HPL GL-1* (Savchenko *et al.*, 2017). Plants were grown in a 14 h light/10 h dark cycle at 22 °C and a light intensity of 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. To account for the diurnal cycle, tissues used for metabolite measurements were collected at the same time of the day (at noon).

Plant treatment and sample preparation

Waterlogging and submergence tolerance (according to the terminology and procedure described in Sasidharan *et al.*, 2017) were studied on plants with an altered oxylipin profile (Table 1). Waterlogging experiments were

performed on 3-week-old seedlings (8–10 leaf stage, before bolting) (see Supplemental Fig. S1 at JXB online). Plants from various genotypes were individually grown in pots filled with ~150 ml of soil (Sunshine Mix 1). Fifteen to twenty-five plants of each genotype were subjected to waterlogging in each experiment. Plants of different genotypes were randomly distributed over each tray in the growth chamber (32 pots with plants per tray). Plants were watered twice a week, wherein 1/5 Hoagland's No. 2 Basal Salt Mix solution, containing $\text{NH}_4\text{H}_2\text{PO}_4$ (23.01 mg l^{-1}), H_3BO_3 (0.57 mg l^{-1}), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (131.28 mg l^{-1}), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (16 $\mu\text{g l}^{-1}$), $\text{Fe}_2(\text{C}_4\text{H}_4\text{O}_6)_3$ (1.06 mg l^{-1}), MgSO_4 (48.15 mg l^{-1}), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.362 mg l^{-1}), MoO_3 (3.2 $\mu\text{g l}^{-1}$), KNO_3 (121.32 mg l^{-1}), and $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (44 $\mu\text{g l}^{-1}$), was used every other watering (Hoagland and Arnon, 1950). Water (or nutrient salts solution) was poured into trays, and excess water was removed from trays after 15 min. To waterlog, pots were subsequently covered with water up to the soil surface level for 10 d, followed by the removal of excess water to allow a 1 week recovery prior to sample collection and fresh weight measurements. In order to prevent a deficiency in mineral nutrition in the soil during the whole period of

Table 1. List of Arabidopsis genotypes used in the study

Analysis	Genotype	Genetic background	AOS		HPL	
			Enzyme	Promoter	Enzyme	Promoter
Oxylipin analysis	Ler	Wild-type Ler	Present	Native	Present	Native
	Col ^a	Wild-type Col	Present	Native	Absent	–
	AOS HPL ^b	Col	Present	Native	Present	Constitutive
Waterlogging tolerance tests and metabolite profiling	AOS hpl gl-1	gl-1 Col	Present	Native	Absent	–
	aos hpl gl-1	gl-1 Col	Absent	–	Absent	–
	aos HPL gl-1	gl-1 Col	Absent	–	Present	Constitutive
	AOS hpl GL-1 ^a	GL-1 Col	Present	Native	Absent	–
	aos hpl GL-1	GL-1 Col	Absent	–	Absent	–
	aos HPL GL-1	GL-1 Col	Absent	–	Present	Constitutive
	AOS HPL GL-1 ^b	GL-1 Col	Present	Native	Present	Constitutive

^aCol and AOS hpl GL-1 – the same line.
^bAOS HPL and AOS HPL GL-1 – the same line

waterlogging, the water level was maintained by regular addition of solution with macro- and micronutrient salts (1/5 Hoagland’s No. 2 Basal Salt Mix solution) to the tray. The watering regime of control plants in other trays remained unchanged. Data are obtained in two independent experiments.

The submergence survival test was performed under similar conditions. For submergence treatment, the water level was maintained at ~10 cm above the soil surface to ensure that the entire seedlings are completely covered by water. The light regime 14 h light/10 h dark cycle was maintained during submergence treatment and the recovery period. The submergence treatment lasted 10 d, and the plant survival rate was scored 7 d after desubmergence. Twenty-four plants of each genotype were analyzed in four independent experiments. Results were expressed as a percentage of surviving plants.

Metabolite profiling was conducted on rosette leaves collected on the fifth day of waterlogging. The tissues were flash-frozen in liquid nitrogen and stored at –80 °C until use. Freeze-dried samples were used to analyze metabolites.

Anthocyanin measurements

The level of anthocyanins was measured in leaf tissue of seedlings at 10 d post-waterlogging. Analyses were carried out as described previously (Mita et al., 1997). The absorption spectrum was measured at λ₅₃₀ and λ₆₅₇ using a UV 1800 spectrophotometer (Shimadzu Co., Tokyo, Japan). Anthocyanin levels were calculated using the (A₅₃₀–0.25×A₆₅₇) equation, and results were expressed as (A₅₃₀–0.25×A₆₅₇) g^{–1} FW.

Chlorophyll fluorescence measurement

Light-induced chlorophyll fluorescence measurements were carried out on fully expanded leaves of growing Arabidopsis plants using a FluorPen FP 100 fluorimeter (Photon Systems Instruments, Czech Republic). PSII operating efficiency was measured in light-adapted samples according to the FluorPen FP 100 fluorimeter manual and calculated according to the equation: Y(II)=(F_m’–F_s)/F_m’, where F_s is the stationary level and F_m’ is the light-induced maximum level of chlorophyll fluorescence in light-adapted leaves as described (Genty et al., 1996).

Measurements of thiobarbituric acid-reactive substances (TBARS)

To measure TBARS, 0.2 g of leaf tissue ground into powder with liquid nitrogen was homogenized in 1 ml of 0.5% (w/v) trichloroacetic acid containing 0.01% butylated hydroxytoluene (Sigma-Aldrich) as an antioxidant. Samples were incubated on ice for 20 min, and then centrifuged at 10 000 g for 15 min at 4 °C. Next, 0.4 ml of

supernatant was mixed well with 0.6 ml of 20% trichloroacetic acid containing 0.65% thiobarbituric acid (TBA) and 0.01% of butylated hydroxytoluene, followed by sample incubation at 95 °C for 30 min, quick cooling on ice, and then centrifugation at 10 000 g for 15 min. The absorbance spectrum of the supernatant was measured at 532 nm with a UV 1800 spectrophotometer (Shimadzu Co.) and corrected for unspecific turbidity by subtracting the value of absorbance at 600 nm and 440 nm, as described in Hodges et al. (1999) with corrections described in Landi (2017). The concentration of TBARS was expressed in μmol g^{–1} FW using an extinction coefficient of 155 mM^{–1} cm^{–1}.

Electrolyte leakage

Ten freshly cut leaf discs (0.5 cm² each) were rinsed with deionized water and floated on 10 ml of deionized water. The conductivity of the solution was measured after 20 h of the leaf discs floating at room temperature using a conductivity meter OK-102/1 (Radelkis, Hungary). Total conductivity was obtained after keeping the flasks in an oven at 90 °C for 2 h. Results were expressed as a percentage of total conductivity.

Metabolite profiling

Extraction and quantification of AOS and HPL branch metabolites were performed as described in Savchenko et al. (2010, 2013).

LC coupled to MS was used to quantify 75 intermediates of central metabolism, including organic acids, nucleotides, phosphorylated compounds, and sugars, as detailed earlier (Schwender et al., 2015). In brief, metabolites were extracted from freeze-dried, pulverized samples with a mixture of chloroform/methanol/water. The resulting methanol/water phase was aliquoted and analyzed using both anion exchange chromatography (ICS-3000, Thermo-Fisher, Dreieich, Germany) and hydrophilic interaction chromatography (Ultimate 3000, Thermo Fisher) coupled to a triple quad mass spectrometer (ABI4000, SCIEX, Germany) using multiple reaction monitoring. All settings for chromatography (including retention times for each individual metabolite) and MS are given in Schwender et al. (2015). Compound identities were verified by mass and retention time match to authenticated standards. External calibration was applied using authenticated standards.

Statistical analysis

Statistical analysis (ANOVA and Cohan’s distance) was done using the software Matlab (R2017b, Mathworks, Ismaning, Germany). Differences between means of sample groups were regarded as statistically significant at P<0.05 and effect size >0.3 (Cohan’s d>0.3, Cohan’s R²>0.2).

Results

Waterlogging-induced alteration of oxylipin profiles

AOS- and HPL-derived metabolites were measured in rosette leaves of plants of different genotypes grown under control and waterlogged conditions (Table 1). The genotypes include *Ler*, a background with functional AOS and HPL genes, and *Col*, the genotype lacking a functional HPL pathway as the result of a deletion in the first exon of the *HPL* gene (Duan *et al.*, 2005), as well as *Col* lines compensated for the absence of HPL by overexpression of rice *HPL3* (*AOS HPL*) (Chehab *et al.*, 2006). The results clearly indicate the expected absence of HPL-derived metabolites in *Col*, and enhanced *cis*-/*trans*-hexenal and *cis*-hexenol in waterlogged *Ler* plants as compared with the respective control (Fig. 1B–D). The increase in *cis*-hexenol is also significant in waterlogged *AOS HPL* plants. Interestingly, waterlogging-mediated alteration of AOS-derived metabolites was limited to increased 12-OPDA, the precursor of JA, in *Col* and *AOS HPL* lines, but not in the *Ler* background. Moreover, these alterations in 12-OPDA levels did not extend to increased JA levels, thus raising the question concerning the mechanism(s) involved in interfering with JA production in spite of enhanced levels of 12-OPDA (Fig. 1E, F).

Genotypes with functional HPL and AOS branches of the oxylipin biosynthesis pathway display enhanced tolerance to waterlogging

To examine the potential role of AOS- and HPL-derived metabolites in waterlogging tolerance, we employed a range of transgenic *Arabidopsis* lines, including those depleted of one or both branches (*aos hpl*, *AOS hpl*, *aos HPL*, and *AOS HPL*) generated in the WT (*GL-1*) or trichomeless (*gl-1*) background of the *Col* accession (listed in Table 1). Initially *aos hpl* and *aos HPL* genotypes were only available in the *gl-1* background, and functional *AOS HPL* genes were only accessible in the *GL-1* background. These lines were employed in the waterlogging stress tolerance study and metabolite analysis. In preliminary experiments, it was established that 10 d waterlogging treatment results in a significant decrease in rosette weight in comparison with control plants; thus, in all experiments, plants remained waterlogged for 10 d. The most notable differential growth phenotypes amongst the genotypes were observed after subsequent recovery after the 10 d waterlogging treatment. Rosette fresh weight of waterlogging-treated plants with functional HPL or AOS branches of the oxylipin pathway was greater than that of plants lacking these branches (Fig. 2A, B; Supplementary Fig. S2). In addition measurements of anthocyanins as an indicator of oxidative stress (Nakabayashi *et al.*, 2014) clearly show that in response to waterlogging as compared with other genotypes, the *HPL*-expressing plants accumulate significantly less anthocyanins in both backgrounds (*gl-1* and *GL-1*) irrespective of the presence or absence of a functional AOS pathway (Fig. 2C).

Mutation in the *GL-1* gene coding for MYB-like protein causes a glabrous trichome phenotype that enhances a plant's susceptibility to environmental stresses (Nambara *et al.*,

Fig. 2. Oxylipin-mediated altered *Arabidopsis* tolerance to waterlogging. Representative images of seedlings of different genotypes challenged with waterlogging for 10 d followed by 7 d of recovery (A), and their corresponding growth by rosette fresh weight (normalized to control) (B). Means $\pm$ SE of 30–50 measurements performed in two independent experiments for each genotype are presented. The average rosette weights of control plants were as follow: *aos hpl gl-1*, 1.676 g; *AOS hpl gl-1*, 0.909 g; *aos HPL gl-1*, 1.236 g; *AOS hpl GL-1*, 1.179 g; *AOS HPL GL-1*, 1.188 g. (C) Anthocyanin levels in leaf tissue measured on the 10th day post-waterlogging and expressed as $(A_{530} - 0.25 \times A_{657}) \text{ g}^{-1} \text{ FW}$. Anthocyanin measurements were performed on three independent samples with 10 plants each. Letters above the bars indicate a statistically significant difference between different genotypes ($P \leq 0.05$).

1998; Yan *et al.*, 2012). Therefore, we generated *aos hpl GL-1* and *aos HPL GL-1* transgenic lines, and tested their stress responses during waterlogging and after reoxygenation (Fig. 3). Specifically, the degree of membrane damage in plant leaves before waterlogging, in waterlogged plants, as well during recovery was evaluated through electrolyte leakage (EL) measurements and the TBARS test (indicative of lipid peroxidation; see Halliwell and Whiteman, 2004). These analyses showed that the difference between genotypes manifests itself only after reoxygenation. The results further indicate that while the integrity of cell membranes in plants growing under waterlogging conditions is not disrupted, membranes are significantly damaged upon reoxygenation, albeit at different levels in various genotypes as reflected by the EL values (Fig. 3A). Indeed,

Fig. 3. Altered plant tolerance to waterlogging and reoxygenation by AOS and HPL branches of the oxylipin biosynthesis pathway. Electrolyte leakage measurements as a proxy for membrane damage (A) and TBARS test as a measure of peroxidative lipid damage (B) in leaves of genotypes with an altered oxylipin profile growing under control conditions (white bars), waterlogged for 5 d (gray bars), and 7 d after reoxygenation (black bars). Means $\pm$ SD of four biological and three experimental replicates are presented. Letters above the bars indicate a statistically significant difference between different genotypes ($P \leq 0.05$). (C) Photosynthetic activity of different genotypes subjected to waterlogging stress followed by reoxygenation evaluated by means of light-induced chlorophyll fluorescence measurements. Means $\pm$ SE of 20–30 measurements are presented. Letters next to line graphs indicate a statistically significant difference between different genotypes in the course of 5 d post-reoxygenation ($P \leq 0.05$).

the EL values were lowest in plants with functional AOS and HPL branches after reoxygenation, indicating the importance of these two branches in maintaining the membrane integrity. In addition, the data show a functional hierarchy of the two branches by displaying higher membrane integrity in the presence of a functional AOS branch compared with the HPL branch of oxylipin biosynthesis. An additional analysis shows similarly increased TBARS levels in leaves of all waterlogged

genotypes examined (Fig. 3B). However, increased TBARS levels in response to reoxygenation are most prominent in plants lacking both branches, and the least prominent in plants with both HPL and AOS branches (Fig. 3B).

Next, we examined the photochemical activity of PSII throughout the waterlogging treatment and after reoxygenation (Fig. 3C). These data show a reduction in PSII activity on the fifth day of waterlogging treatment, with a notable decline upon reoxygenation at day 10 and thereafter. Amongst all the examined genotypes, those with functional HPL and AOS showed the least decline in PSII in response to reoxygenation.

Moreover, the various genotypes in the *GL-1* background were analyzed for their tolerance to submergence (see Supplemental Fig. S3). The submergence survival test performed on these lines supported the earlier finding and confirmed that plants expressing both AOS and HPL genes displayed the highest survival rate of ~77%, whereas under the same conditions the survival rate of *aos hpl* lines was only ~30%, and that of *AOS hpl* and *aos HPL* plants was ~50%. The results clearly demonstrate that the presence of either the AOS or HPL branch improves plant performance in response to waterlogging stress and submergence. However, the presence of both functional branches further improves plant stress tolerance.

Central metabolites are altered by AOS- and HPL-derived oxylipins

Next, we examined waterlogging-mediated alteration in the overall metabolic profile of various genotypes differing by the presence/absence of the functional AOS and HPL branches (Figs 4–6; Supplementary Table S1). All genotypes showed shifts in a similar set of metabolites: statistically significant increases in sucrose, trehalose-6-phosphate, myo-inositol, and related raffinose family oligosaccharides (RFOs) (raffinose, stachyose, verbascose), succinate, Ser, Pro, Tyr, and Leu; consistent decreases in UDP-glucose, hexose phosphates, citrulline, Asp, Met, intermediates of the Met salvage pathway, NAD, NADP, and ascorbate. Several lines showed a decrease in Ala and lactate (statistical analysis of the data is provided in Supplementary Fig. S4). These alterations were most notable in *aos hpl gl-1* plants, reflecting the higher sensitivity of this genotype to waterlogging. In fact, these plants are characterized by a much stronger accumulation of major sugars (sucrose, glucose, and fructose), organic acids related to the tricarboxylic acid (TCA) cycle, and more numerous changes in energy metabolites (nucleotides and redox equivalents) (Fig. 4; Supplementary Fig. S5). Correlation analysis of these data revealed a link between the build up of sucrose and the accumulation of hexoses, RFOs, glycolytic intermediates, the sugar signal trehalose-6-phosphate, and selected acids of the TCA cycle. Altogether, the metabolic alterations seen in the studied genotypes are similar to previous reports on waterlogging-induced stress responses (Obata and Fernie, 2012).

The knockout mutant *aos* was found to differ significantly in its metabolite profile from AOS-expressing plants (both in the *gl-1* background) (Fig. 5; Supplementary Figs S6, S7). Principal component analysis of plants grown under control conditions showed that both genotypes form clearly distinct clusters; this

Fig. 4. Response of the *AOS hpl gl-1* and *AOS hpl GL-1* plants to waterlogging treatment at the level of individual metabolites. Colors represent positive (red color box) and negative (blue color box) correlation between sucrose and metabolites of central metabolism (Pearson's product-moment correlation) in control and waterlogged plants; bold framing indicates statistical significance at $P < 0.05$. Letters above the bars indicate a statistically significant difference between different genotypes and treatments ($P \leq 0.05$).

Fig. 5. Metabolic profiles of leaf tissues of various genotypes under control and waterlogging conditions, illustrating differences between the contribution of AOS and HPL branches to the level of individual metabolites in response to waterlogging treatment. Letters above the bars indicate a statistically significant difference between different genotypes and treatments ($P \leq 0.05$).

clustering was based on the build up of sucrose, fructose, and glucose, while hexose-P and several glycolytic intermediates (mainly Trp) were depleted in the mutant. UDP-gluc, ADP-gluc, and Met intermediates were also decreased, which possibly

reflects lower pathway activities for sucrose cleavage, hexose activation (phosphorylation), starch biosynthesis, and the Met salvage pathway. In addition, shikimate pathway-derived amino acids [starting from phosphoenolpyruvate (PEP)] and several

Fig. 6. HPL-dependent regulation of central metabolism under control and waterlogging conditions. Alteration in the levels of individual metabolites in plants with and without a functional HPL branch grown under control and waterlogging conditions. Letters above the bars indicate a statistically significant difference between different genotypes and treatments ($P \leq 0.05$).

other amino acids were lower, ascorbate and polyamines were depleted, and five acids of the TCA cycle accumulated. Under waterlogging conditions, clusters of the two genotypes (*AOS hpl gl-1* and *aos hpl gl-1*) were to some extent overlapping, indicating some similarity in the metabolic response (Fig. 5; Supplementary Fig. S6). However, ascorbate was fully depleted in the mutant, as also seen for NAD and NADP (Fig. 5; Supplementary Fig. S7). This is indicative of a severe shift in redox-related processes, which probably contributes to the higher susceptibility of the mutant under waterlogging stress (see above).

Interestingly, the majority of changes observed in AOS-depleted plants were completely or partially alleviated in *HPL*-overexpressing plants (*aos HPL* plants in the *gl-1* background) growing under control conditions (Fig. 5; Supplementary Fig. S8). This was especially obvious for hexose-phosphates, Asp-derived amino acids, Met salvage metabolites, and ascorbate. Notably, under waterlogging conditions, the majority of metabolites in *aos HPL gl-1* plants returned to the non-stressed *AOS hpl gl-1* level. This was evident for sucrose, hexoses, some glycolytic intermediates and acids of the TCA cycle, several amino acids, and the sugar signaling molecule trehalose-6-phosphate. This might contribute to the fact that *aos HPL gl-1* plants also showed an improved waterlogging tolerance (see above).

The *HPL*-dependent regulation of metabolism was further investigated by analyzing *HPL*-overexpressing plants in the *GL-1* background (WT Col) (Fig. 6; Supplementary Figs S9, S10). Principal component analysis revealed that the metabolite pattern in the *HPL* overexpressors was distinct from that of the corresponding control plants lacking a functional *HPL* branch, under both control and (especially) waterlogging conditions (Supplementary Fig. S10). The two independent transgenic lines overexpressing the rice *HPL3* gene (*AOS HPL GL-1* plants) showed largely consistent changes compared with *AOS hpl GL-1* plants under standard growth conditions, in particular, an accumulation of sucrose and RFOs, shifts in redox-related compounds including polyamines (citrulline, ornithine, and numerous changes in energy metabolites (Fig. 6; Supplementary Fig. S10). In addition, some glycolytic intermediates and transport-related amino acids (Asn, Glu) were affected. Under waterlogging conditions, the most striking differences between transgenics and WT Col were seen for ascorbate and glutathione (strongly depleted), polyamines (approximately half the pool size), and NAD/NADH/NADPH (approximately a third), which altogether hints at severe shifts in redox metabolism under stress treatment. Moreover, all intermediates of the Met salvage pathway were significantly reduced in both transgenic lines as compared with *AOS hpl GL-1* plants. Finally, we observed significant reductions for Asp-derived amino acids, while those of the shikimate pathway (Trp, Tyr, and Phe) and some branched-chain amino acids (Leu) were consistently elevated in the transgenic plants.

Discussion

Oxylipins mediate enhanced tolerance to waterlogging

Chloroplast-localized AOS and *HPL* enzymes of parallel branches of the oxylipin biosynthesis pathway compete for the

common substrate, 13-HPOT, formed in plastids (Tong et al., 2012; Savchenko et al., 2014a; Nilsson et al., 2016). Our data show a waterlogging-induced increase in selected oxylipins, namely enhanced levels of *HPL*-derived metabolites in all examined genotypes with functional *HPL*. Interestingly, the enhanced levels of the JA precursor, 12-OPDA, is only observed in Col backgrounds that either lack a functional *HPL* or are complemented by constitutive expression of the *HPL* gene from rice (Chehab et al., 2006). The difference between WT Ler and *AOS HPL* in the Col background could potentially be due to the aberrant expression of *HPL* under the 35S promoter, or a difference between enzyme activity of rice *HPL* versus native Arabidopsis *HPL*, and thus the availability of 13-HPOT to the AOS pathway for 12-OPDA production. Interestingly, none of the lines examined produced any detectable JA above basal levels in response to waterlogging. These data corroborate those previously reported in rice (Lu et al., 2015). The accumulation of the precursor rather than the final product, JA, potentially implies that translocation of 12-OPDA from the chloroplast to the peroxisome, the site of β -oxidation for JA production, might be compromised in waterlogged plants. Alternatively, the β -oxidation pathway might function inefficiently in the waterlogged plants. Lack of JA accumulation, however, does not imply an absence of 12-OPDA-mediated signal transduction, as this molecule is an active signal molecule that not only up-regulates COI1-dependent genes that are also regulated by JA, but also is capable of inducing, in a COI1-independent fashion, genes that are not induced by JA, as well as regulating the expression of genes in a COI1-dependent manner albeit independently of JA (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008; Lemos et al., 2016).

Combined approaches of stress survival test, measurements of biomass accumulation, fatty acid peroxidation, cell membrane integrity, PSII activity, and anthocyanin accumulation in various submerged or waterlogged genotypes show the potency of each of the *HPL* and AOS branches in enhancing tolerance, although most notably when combined. Analyses of available phenotypes in both *gl-1* and *GL-1* backgrounds revealed a similar protective function of both oxylipin biosynthesis branches. Interestingly, an increase in the level of oxidized fatty acids and a decrease in PSII activity are observed in waterlogged plants, while cell membrane damage, manifested as an increase in EL, became obvious only after reoxygenation. The ability to maintain photosynthesis and membrane integrity strongly correlates with the tolerance of plants to waterlogging stress (Ren et al., 2016).

It is reported that JA biosynthesis- and signaling-deficient mutants are more sensitive to reoxygenation, whereas transgenic lines overexpressing the JA-regulated transcription factor gene *MYC2* are more tolerant to post-hypoxic stress (Yuan et al., 2017). In fact, application of exogenous methyl jasmonate improved tolerance of wild-type Arabidopsis plants to reoxygenation (Yuan et al., 2017). In our study, the *aos* mutant lines incapable of producing AOS-derived metabolites displayed the least tolerance to submergence and waterlogging. The unchanged levels of JA in waterlogged backgrounds with active AOS genes therefore suggest that enhanced tolerance to reoxygenation is driven either solely by 12-OPDA or in conjunction with the basal JA levels present.

The observed increase in anthocyanin levels of all genotypes when waterlogged corroborates the stress-induced accumulation of this pigment (Kazan and Manners, 2011). However, significant accumulation of anthocyanins in *aos* mutant plants does not support the notion of a JA-dependent accumulation of the pigment (Qi *et al.*, 2011). Interestingly, however, the presence of a functional *HPL* significantly reduces the levels of the enhanced anthocyanins in response to waterlogging. This suggests either that *HPL*-expressing plants are not as severely stressed or that the *HPL* branch somehow interferes with anthocyanin production.

Metabolic adaptation to stagnant waterlogging

Significant waterlogging-induced alterations of metabolite pool sizes in leaves of the studied plants indicate that above-ground organs of waterlogged plants experience stress. Some alterations, including an increase in the levels of sucrose, initial products of sucrose hydrolysis, trehalose-6-phosphate, RFOs, and *myo*-inositol, the biosynthetic substrate for RFOs biosynthesis, can be described as a well-known general stress response. RFOs are osmoprotectants, stabilizers of cellular membranes, and scavengers of hydroxyl radicals (Nishizawa *et al.*, 2008), known to accumulate in response to stresses that give rise to excess concentrations of reactive oxygen species (Cook *et al.*, 2004; Urano *et al.*, 2009). Inositol is an important metabolic and signaling compound, regulating cell–cell communication, availability of active auxin (Chen and Xiong, 2010), and responses to salt and dehydration stress (Nelson *et al.*, 1998). In the TCA cycle, an accumulation of succinate was a major alteration in most stressed plants, which is also a very common plant response to environmental stresses (Doncheva *et al.*, 2006). The most noticeable alteration of the amino acid profile was an increase in the levels of the stress response metabolites Pro and Ser (Rai, 2002) and a decrease in oxaloacetate family amino acid levels, including Asp and Asp-derived Asn. Met and Met-related amino acids are also significantly decreased in leaves of all waterlogged plants, probably as a result of S and N deficiency or intensified consumption of Met for ethylene production through S-adenosylmethionine (Ravanel *et al.*, 1998). It is known that flooding/waterlogging stresses are accompanied by the production of ethylene, which regulates many adaptive responses under these stress conditions (Sasidharan and Voesenek, 2015). Met can also be consumed in the biosynthesis of polyamines, and in crucifers in the production of the specialized metabolites glucosinolates. A strong decrease in the citrulline level after waterlogging is obvious in all lines. Citrulline is an efficient hydroxyl radical scavenger and a strong antioxidant; this metabolite accumulation correlates with tolerance to salt and drought stress (Akashi *et al.*, 2001; Kusvuran *et al.*, 2013).

Virtually all biotic and abiotic stresses are accompanied by oxidative stress, and the ability to detoxify activated oxygen species is related to a higher stress tolerance (Yeung *et al.*, 2018). Ascorbate (Asa) and reduced glutathione (GSH) are the main antioxidant components present in plant cells. In our experiments, a decrease in Asa was obvious even in relatively tolerant plants, while no significant alteration in GSH level occurred. This confirms active work of the antioxidant system

and an important role for Asa in adaptation to waterlogging conditions. The level of NAD and NADP, playing a central role in maintaining plant energy status and redox homeostasis (Hashida *et al.*, 2009), decreased significantly in all waterlogged plants.

Oxygen limitation generally induces characteristic metabolic changes such as induction of anaerobic metabolism, including the formation of acetaldehyde and ethanol, accumulation of pyruvate and some organic acids, and lowering of cytoplasmic pH (Borisjuk and Rolletschek, 2009; van Dongen *et al.*, 2009; Mustroph *et al.*, 2014; van Veen *et al.*, 2016). Some species delay or avoid accumulation of ethanol by diverting glycolytic intermediates to alternative end-products such as lactate, malate, succinate, γ -aminobutyrate (GABA), and Ala (Shingaki-Wells *et al.*, 2011). We found that in non-flooded leaves of waterlogged plants, Ala and lactate levels decreased and the GABA level remained unchanged, suggesting that accumulation of these metabolites is indeed an indication of oxygen deficiency.

Oxylipin pathway-dependent characteristics of the metabolite pattern

According to waterlogging tolerance tests, the difference between plants of the studied genotypes become noticeable only after reoxygenation, but analysis of central metabolites revealed significant differences between these plants growing under normal conditions and undergoing waterlogging. A decrease in amino acids, depletion of AsA and polyamines, and accumulation of TCA cycle acids indicate a significant alteration of metabolism in *aos* plants, including altered glycolysis, disturbance of the redox status, and shifts in respiratory activity (Fig. 5; Supplementary Fig. S7). The *aos* mutant lines show a build up of sucrose while hexose-P and most glycolytic intermediates (but not pyruvate) are depleted. Lower levels of UDP-glucose, ADP-glucose, and Met intermediates allow the assumption of lower pathway activities for sucrose cleavage, hexose activation, starch biosynthesis, and Met salvage. The level of 3-phosphoglycerate (3-PGA) is very low in *aos hpl gl-1* plants [the 3-PGA/dihydroxyacetone phosphate (DHAP)+glyceraldehyde 3-phosphate (G3P) ratio is smallest in these plants], and waterlogged *aos hpl gl-1* do not accumulate Ser, synthesized from 3-PGA, unlike plants with a functional AOS branch. In the TCA cycle, the level of 2-oxoglutarate is lower in *aos* plants, while aconitate and isocitrate accumulate in leaf tissues. The level of Val, accumulation of which was shown in connection to the response to elevated CO₂ concentration in drought-stressed plants, drought stress tolerance, cold shock, and heat shock (Kaplan *et al.*, 2004; Merewitz *et al.*, 2012), is also decreased in *aos* plants. Another pathway potentially regulated by jasmonates is the formation of Asp and Asp-derived amino acids, including Ile, Thr, Met, and Met salvage metabolites. There is a significant decrease in S-containing metabolites, in accordance with the previously reported connection between jasmonates and sulfur metabolism (Jost *et al.*, 2005; Kopriva, 2013; Park *et al.*, 2013). Plants depleted of the AOS branch have increased levels of orotate, a metabolite possessing growth-stimulating activity (Shopova and Moskova-Simeonova, 2000). The level of antioxidants, important for

alleviation of oxidative damage caused during stresses, is also very low in plants depleted of jasmonates, confirming that JA signaling interacts with the antioxidant pathway. Even under normal conditions, metabolism of *aos* plants in many aspects resemble metabolism of plants experiencing oxidative stress. Stress-induced alterations in the metabolite profile are more pronounced in *aos* plants, but, in general, the mutant under stress conditions is more similar to the WT than under standard growth conditions. So, according to the data obtained, the AOS branch is involved in regulation of carbohydrates, amino acids, energy metabolites, and components of the antioxidant system.

The presence of a functional HPL enzyme in leaf tissue partially 'complements' the *aos* knockout chemotype. Plants overexpressing the *HPL* gene in the *aos* background (*aos HPL gl-1* plants) display a profile of metabolites more similar to *AOS hpl gl-1*. Further studies are needed to find out if the observed effect is a result of overlapping functions of AOS and HPL branch metabolites or whether both pathways efficiently eliminate the common toxic 13-HPOT substrate.

In addition to overlapping functions, it is obvious that the two branches have distinct functions. A decrease in adenine and Ile levels caused by the absence of the AOS branch is not alleviated by *HPL* expression. Moreover, the activity of both studied branches has opposite effects on sucrose levels. The sucrose level is higher in plants overexpressing *HPL* grown under normal conditions, irrespective of the presence or absence of a functional AOS gene, but glucose and fructose levels are lower or not altered. RFOs are also higher in *HPL*-expressing lines. Interestingly, while in other genotypes the glucose level is increased after waterlogging, in *HPL*-expressing plants it is not so obvious. The most striking differences of transgenic plants versus plants depleted of the HPL branch are seen for ascorbate, which is fully depleted in transgenic lines, and a significant decrease in GSSG, polyamines, and NAD/NADH/NADPH, which altogether hint at shifts in redox metabolism under stress treatment. In general, all waterlogging-induced alterations of metabolism are less pronounced in more tolerant *HPL*-expressing plants. A known general stress response, the accumulation of succinate, was not displayed in very tolerant *AOS HPL* plants in the *GL-1* background under waterlogging conditions, in contrast to more susceptible plants, leading to the conclusion that this metabolite is indeed a good stress indicator. Waterlogged *HPL*-expressing plants accumulate more branched-chain and aromatic amino acids, specifically Tyr and Phe, and significantly less anthocyanins. Previously the plasticity of the phenylpropanoid pathway derived from Phe and a connection between anthocyanin biosynthesis and phenylpropanoid metabolites have been demonstrated (Stracke et al., 2009; Qi et al., 2011; Laursen et al., 2015).

In summary, here we show that under waterlogging conditions, both AOS and HPL pathways contribute to the adaptation process. We establish broad genotype-specific alterations of metabolite profiles, thereby unmasking the new role of oxylipins in the regulation of plant primary metabolism, and describe the individual and overlapping functions of AOS and HPL. The role of the HPL branch in regulation of primary

metabolism and tolerance to waterlogging stress is demonstrated for the first time. Molecular mechanisms underlying metabolic changes in the studied genotypes with an altered oxylipin profile remain unknown. It is most likely that the oxylipins implement regulatory functions through various signal transduction pathways. Such a mechanism has been shown for the co-ordinated regulation of primary metabolic pathways and secondary metabolism through the single jasmonate-responsive transcription factor ORCA3 in *Catharanthus roseus* (van der Fits and Memelink, 2000). The results presented here provide a basis for the identification of the molecular components of the oxylipin-mediated signaling pathways controlling the levels of individual metabolites. These data will be instrumental for better understanding of the link between secondary and primary metabolism and their collective impact on plant stress tolerance.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Complete metabolite data (Excel format).

Fig. S1. Representative trays with studied plants before, during, and after waterlogging.

Fig. S2. Effect of waterlogging stress on plants with an altered oxylipin profile.

Fig. S3. Effect of submergence stress on plants with altered oxylipin profile.

Fig. S4. Statistical analysis of metabolite profiling.

Fig. S5. Response of the *AOS hpl gl-1* and *AOS hpl GL-1* plants to waterlogging treatment at the level of individual metabolites extracted from leaf tissue.

Fig. S6. Principal component analysis of leaf metabolite data in plants possessing and depleted of a functional AOS branch grown under control and waterlogged conditions.

Fig. S7. AOS-dependent regulation of central metabolism under normal and waterlogging conditions.

Fig. S8. Principal component analysis of metabolite data in leaves of plants possessing or depleted of functional oxylipin branches grown under control and waterlogged conditions.

Fig. S9. HPL-dependent regulation of central metabolism under normal and waterlogging conditions [independent transgenic line (*AOS HPL GL-1* line 2)].

Fig. S10. Principal component analysis of metabolite data of leaves of plants possessing or depleted of a functional HPL branch (two independent transgenic lines) grown under control and waterlogged conditions.

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