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Research Article

Rapid systemic responses of *Arabidopsis* to waterlogging stress

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Abstract

Waterlogging stress (WLS) negatively impacts the growth and yield of crops resulting in heavy losses to agricultural production. Previous studies have revealed that WLS induces a systemic response in shoots that is partially dependent on the plant hormones ethylene and abscisic acid. However, the role of rapid cell-to-cell signaling pathways, such as the reactive oxygen species (ROS) and calcium waves, in systemic responses of plants to WLS is unknown at present. Here, we reveal that an abrupt WLS treatment of *Arabidopsis* (*Arabidopsis thaliana*) plants growing in peat moss triggers systemic ROS and calcium wave responses and that the WLS-triggered ROS wave response of *Arabidopsis* is dependent on the ROS-generating RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD), calcium-permeable channels GLUTAMATE-LIKE RECEPTOR 3.3 and 3.6 (GLR3.3 and GLR3.6), and aquaporin PLASMA MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1) proteins. We further show that WLS is accompanied by a rapid systemic transcriptomic response that is evident as early as 10 min following waterlogging initiation, includes many hypoxia-response transcripts, and is partially dependent on RBOHD. Interestingly, the abrupt WLS of *Arabidopsis* resulted in the triggering of a rapid hydraulic wave response and the transient opening of stomata on leaves. In addition, it induced in plants a heightened state of tolerance to a subsequent submergence stress. Taken together, our findings reveal that the initiation of WLS in plants is accompanied by rapid systemic physiological and transcriptomic responses that involve the ROS, calcium, and hydraulic waves, as well as the induction of hypoxia acclimation mechanisms in systemic tissues.

Introduction

As our climate changes, the frequency and intensity of weather episodes, such as floods and heavy downpours, gradually increase (Bailey-Serres et al. 2019; Masson-Delmotte et al. 2021). Floods and heavy downpours can cover entire fields causing complete or partial submergence of crops and/or create lasting conditions of waterlogging stress (WLS) by soaking the soil with water for extended periods of time (Voesenek and Bailey-Serres 2015; Loreti et al. 2016; Pucciariello and Perata 2017; Sasidharan et al. 2018, 2021). These conditions limit oxygen availability to the root system (waterlogging), or the entire plant (submergence), induce hypoxia- and/or anoxia-response mechanisms, and negatively impact crop growth and yield, resulting in heavy losses to agricultural production (Bailey-Serres et al. 2019).

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Waterlogging can rapidly occur under field conditions following a sudden downpour, or as a result of advancing flood water, and create a situation in which the roots are subjected to hypoxia, while the shoots are not (Voesenek and Bailey-Serres 2015). Previous work has shown that waterlogging causes local hypoxia-driven responses in the roots, and systemic responses in the shoots that involve adjustments in carbohydrate metabolism, ubiquitin-dependent protein degradation, hormonal responses, and many other molecular and metabolic responses (Hsu et al. 2011). In addition, some of the systemic responses induced by WLS were found to be altered in mutants deficient in ethylene and abscisic acid (ABA) signaling (Hsu et al. 2011; Tsai et al. 2014).

Among the first responses to anoxia conditions in roots or shoots of plants are the inhibition of mitochondrial respiration, the activation of calcium signaling, and the accumulation of reactive oxygen species (ROS; Voesenek and Bailey-Serres 2015; Loreti et al. 2016; Pucciariello and Perata 2017; Sasidharan et al. 2018, 2021; Yang et al. 2022). Recent studies demonstrated that the vacuolar H^+/calcium transporter CATION/PROTON EXCHANGER 1 (CAX1) and the RESPIRATORY BURST OXIDASE HOMOLOGs D and F (RBOHD and RBOHF) play important roles in these responses and that changes in calcium signaling and ROS are important for triggering different anoxia-response mechanisms, including transcript accumulation, and in some instances aerenchyma formation (Liu et al. 2017; Yang et al. 2022). The function of RBOHD, RBOHF, and CAX1 was also shown to be required for plant acclimation to anoxia stress (Liu et al. 2017; Yang et al. 2022).

As WLS can occur rapidly in the field (e.g. Voesenek and Bailey-Serres 2015), cause rapid calcium- and RBOH-driven ROS production in roots (Liu et al. 2017; Yang et al. 2022), and trigger systemic responses in the shoot (Hsu et al. 2011), we hypothesized that WLS could trigger a rapid systemic signaling response that involves the ROS wave. The ROS wave is a cell-to-cell signaling mechanism that depends on RBOHD and RBOHF function and propagates through the vascular bundles and/or mesophyll cells of plants from the site of its stimulation (by abiotic or biotic stress) to the entire plant within minutes (Zandalinas, Fichman, Devireddy, et al. 2020; Zandalinas, Fichman, and Mittler 2020; Fichman and Mittler 2021; Fichman et al. 2022; Mittler et al. 2022). Integrated with the calcium and electric waves, the ROS wave is also required for the activation of many molecular, physiological, and metabolic responses of systemic tissues, as well as the overall acclimation of plants to different stresses (e.g. Kollist et al. 2019; Fichman, Zandalinas, and Sengupta, et al. 2020).

Here, we show that an abrupt WLS treatment of *Arabidopsis* (*Arabidopsis thaliana*) plants (root system flooding) growing in peat moss triggers a systemic (shoots and leaves) ROS and calcium wave responses and that the WLS-triggered ROS wave response of *Arabidopsis* is dependent on RBOHD, the calcium-permeable channels GLUTAMATE-LIKE RECEPTOR 3.3 and 3.6 (GLR3.3 and GLR3.6), and the aquaporin/peroxiporin PLASMA MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1). We further show that WLS is accompanied by a rapid systemic transcriptomic response that is partially dependent on RBOHD. Interestingly, the abrupt WLS of *Arabidopsis* resulted in the triggering of a rapid hydraulic wave response and the transient opening of stomata on leaves, as well as the induction of a heightened state of tolerance to a subsequent submergence stress (of the entire plant). Taken together, our findings reveal that WLS is accompanied by rapid systemic molecular and physiological responses that involve the ROS, calcium, and hydraulic waves and that the ROS wave triggered upon WLS in *Arabidopsis* is dependent on RBOHD, GLR3.3 and GLR3.6, and PIP2;1 function. These findings suggest that systemic (root-to-shoot) plant responses to WLS are rapid and at least partially dependent on cell-to-cell signaling.

Results

Inducing WLS in *Arabidopsis*

To mimic conditions that accompany a sudden event of WLS, caused by a heavy downpour or advancing flood water, we grew *Arabidopsis* plants in peat soil under controlled growth conditions, allowed the water content of the peat soil to reach 54 ± 2% of full water capacity, and subjected plants to WLS by rapidly watering plants until the water level reached all the way to the top of the peat soil (rapidly equilibrate to about 100% water capacity; Fig. 1A). This treatment resulted in a significant decrease in oxygen levels measured with an oxygen electrode, as early as 10 min following WLS initiation (Fig. 1B). The experimental system used in this study resulted therefore in a state in which the top part of the plant remained in the air, while the entire root system of the plant experienced hypoxia stress caused by the WLS treatment (Fig. 1). In addition, it enabled the application of different chemical treatments (e.g. ABA application) prior to WLS application (Fig. 1A; see below).

WLS triggers the systemic ROS and calcium wave responses

Using the experimental system shown in Fig. 1, we studied whether WLS applied to the roots triggers the ROS and calcium waves in the systemic tissues of plants (leaves) subjected to WLS. As shown in Fig. 2, A and B, WLS resulted in the activation of systemic ROS (Fig. 2A) and calcium (Fig. 2B) wave responses that were detected in the upper parts of plants within 10- (calcium) and 20-min (ROS) post-WLS application, respectively (measured using our live whole-plant imaging method; Fichman et al. 2019). To examine whether the ROS wave response detected in plants subjected to WLS resulted in enhanced accumulation of hydrogen peroxide (H_2O_2), which plays a key role in regulating plant responses to stress (Mittler et al. 2022), we also measured H_2O_2 levels in shoots of plants subjected to WLS

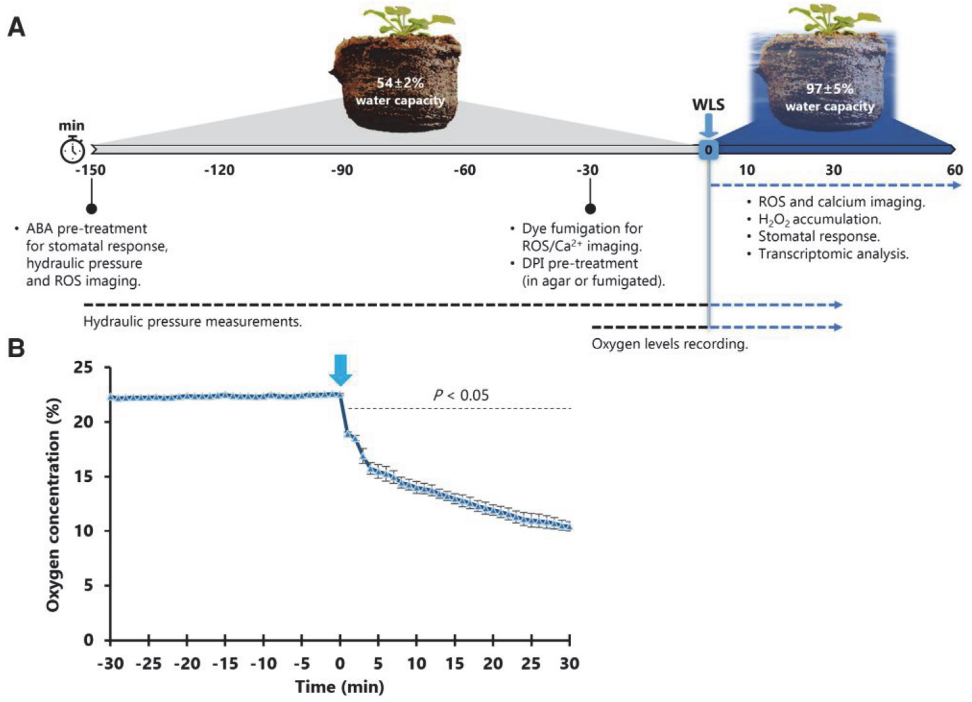

Figure 1. Experimental design and measurements of oxygen levels. **A)** The experimental design used to induce WLS in *Arabidopsis*. Plants grown in peat soil at a defined water content were watered until water reached the top of the peat soil level and analyzed for systemic responses as described in the text. **B)** Measurements of oxygen level at the middle of the peat soil prior to and during the WLS treatment (applied at 0 min). Results are presented as the means ± SE. Statistical analysis was performed with a 2-sided Student's *t* test ($^*P < 0.05$; $n = 3$). CT, control.

using the Amplex Red method (Fichman et al. 2022). In contrast to the whole-plant imaging of ROS with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), that measures general ROS accumulation, the Amplex Red method can measure an increase or decrease in H_2O_2 levels in extracts from cells or tissues (Fichman et al. 2019, 2022). As shown in Fig. 2C, H_2O_2 levels were transiently elevated in shoots of plants subjected to WLS for 30 min and declined at 60-min poststress application.

WLS triggers a systemic hydraulic wave response and causes the transient opening of stomata

Plants respond to different treatments that abruptly alter the water pressure in their vascular system with a hydraulic wave (e.g. wounding; Kloth and Dicke 2022; Gao et al. 2023; Grenzi et al. 2023). As the sudden application of WLS could potentially activate a hydraulic wave in plants due to an increase in the water pressure around the root system, we measured the systemic hydraulic wave in plants subjected to WLS (Zimmermann et al. 2013; Fichman and Mittler 2021). As shown in Fig. 3A, WLS resulted in the triggering of a rapid systemic hydraulic wave response that was detected in shoots within 5 min of WLS application to the root system. Interestingly, the application of a sudden WLS to plants also resulted in a transient stomatal opening response that started at about 1 min following the application of WLS and lasted for about 10 min (Fig. 3B). To examine whether the hydraulic wave (Fig. 3A) and the transient stomatal opening response (Fig. 3B), triggered by WLS, were linked, we pretreated plants with ABA that caused stomata to close and applied WLS. As shown in Fig. 3, A and B, pretreatment of plants with ABA (50 μM) 150 min before WLS suppressed the hydraulic wave response as well as the transient stomatal response of plants to the sudden WLS treatment.

The WLS-triggered ROS wave is dependent on RBOHD, GLR3.3 and GLR3.6, and PIP2;1 function

The systemic ROS wave response of *Arabidopsis* to a local treatment of excess light stress or wounding was previously shown to depend on the function of different proteins such as RBOHD, GLR3.3 and GLR3.6, PLASMODESMATA LOCALIZED PROTEIN 5 (PDLP5), and/or PIP2;1 (Fichman et al. 2021; Fichman and Mittler 2021). To test whether the WLS-triggered ROS wave (Fig. 2A) is also dependent on RBOHD function, we applied a drop of the broad-range oxidase and RBOH inhibitor diphenyleneiodonium (DPI, 50 μM) or water, in agarose, to the middle point between the root system and the shoot (just above the peat soil level, as described in Devireddy et al. 2018), 30 min prior to subjecting plants to WLS. In addition, we compared the response to WLS between wild type (WT) and the *rbohD Arabidopsis* mutant. As shown in Fig. 4, pretreatment of plants with a drop of agar containing DPI prior to WLS (Fig. 4A) or treatment of the *rbohD* mutant (Fig. 4B) with WLS resulted in the suppression of the ROS wave response induced by WLS in

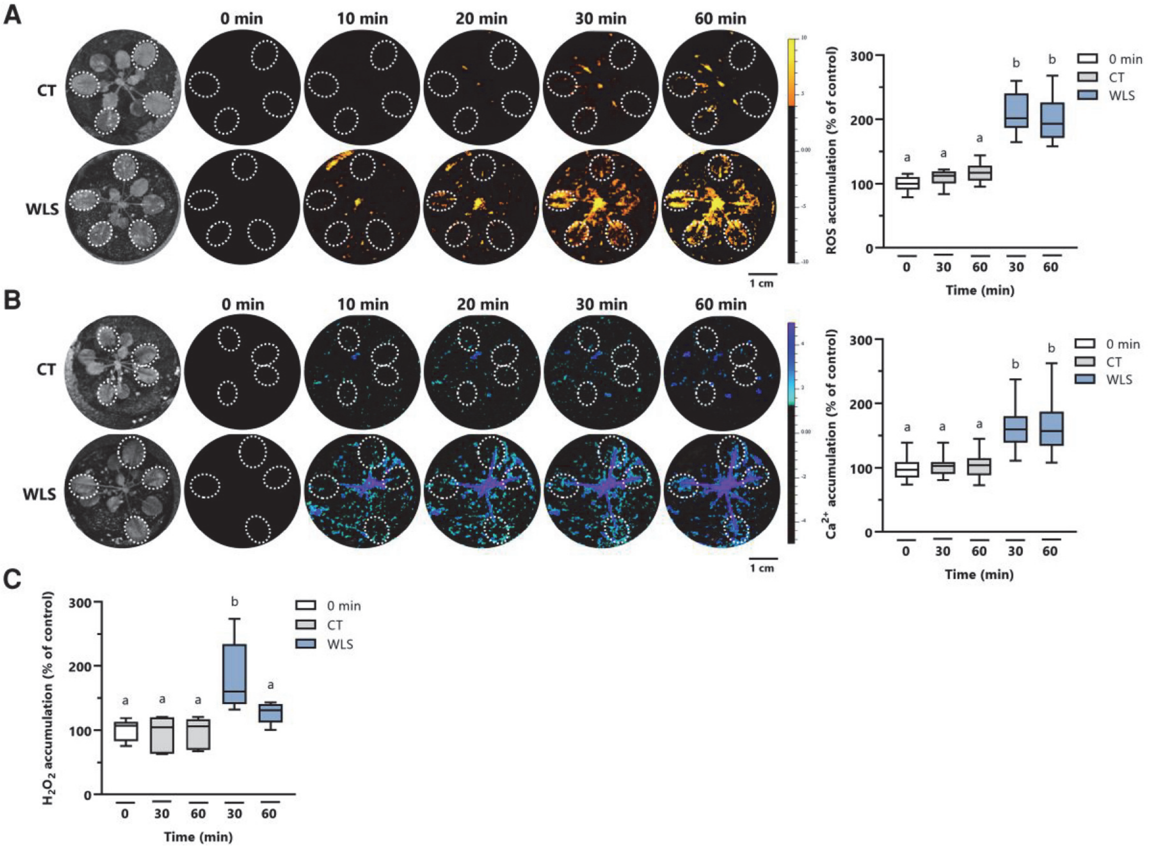

Figure 2. Imaging of systemic ROS and calcium accumulation in WT plants subjected to an abrupt WLS treatment. **A)** *Arabidopsis* plants were fumigated with H_2DCFDA and imaged for systemic ROS accumulation in response to WLS. **B)** Same as in **A)**, but for plants fumigated with Fluo-4-AM and imaged for systemic calcium accumulation. Representative time-lapse images of whole-plant ROS or calcium accumulation in treated and untreated *Arabidopsis* plants are shown alongside box plots of combined data from all plants used for the analysis at the 0- and 30-min time points in **A)** and **B)**. Radiant efficiency values were measured in the regions of interest indicated by ovals in the images using the math tools of Living Image 4.7.2 software. Accumulation of ROS was determined by subtracting the signal of the initial time point (0-min) from the time point of interest. Color scale indicates intensity of calculated ROS **A)** and calcium **B)** accumulation. Magnification bars in **A)** and **B)** are 1 cm. All experiments were repeated at least 3 times with 8 plants per repeat. Data are shown as box and whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Center line corresponds to the median and the whiskers to the maximum and minimum values. Different letters denote significance at $P < 0.05$ (1-way ANOVA followed by Tukey's post hoc test). **C)** H_2O_2 accumulation in *Arabidopsis* plants in response to WLS. Representative data of 6 independent replicates are shown as box and whisker plot with borders corresponding to the 25th and 75th percentiles of the data. Center line corresponds to the median and the whiskers to the maximum and minimum values. Different letters denote significance at $P < 0.05$ (1-way ANOVA followed by a Tukey's post hoc test). CT, control; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate.

Arabidopsis. A similar result was found when whole plants were fumigated with DPI 30 min prior to the application of WLS (Supplemental Fig. S1). These findings suggest that RBHOD function is required for the WLS-induced ROS wave response.

To determine the role of GLR3.3 and GLR3.6, PDLP5, and PIP2;1, previously found to be involved in regulating the ROS wave response to injury or excess light stress (Fichman et al. 2021; Fichman and Mittler 2021), in mediating the WLS-triggered ROS wave response, we subjected WT and *glr3.3glr3.6*, *pdlp5*, *pip2;1*, and *pip1;4* mutants to WLS and measured their systemic ROS wave response. As shown in Fig. 5A, the function of GLR3.3 and GLR3.6 and PIP2;1 was required for the WLS-induced ROS wave response, while the function of PIP1;4 and PDLP5 was not.

As pretreatment of plants with ABA blocked the transient stomatal opening response of plants, as well as the hydraulic wave response, to WLS (Fig. 3), we also tested whether pretreatment with ABA will block the ROS wave in response to WLS. As shown in Fig. 5B, pretreatment of plants with ABA suppressed the ROS wave response to WLS.

Rapid systemic transcriptomic responses to WLS in WT and the *rbohD* mutant

The transcriptomic response of *Arabidopsis* to anoxia, hypoxia, submergence, or simulated WLS was previously studied in *Arabidopsis* (Liu et al. 2005; Hsu et al. 2011; Lee et al. 2011; Licausi et al. 2011; Pucciariello et al. 2012; Hsu et al. 2013; Tsai et al. 2014; van Veen et al. 2016; Giuntoli et al. 2017;

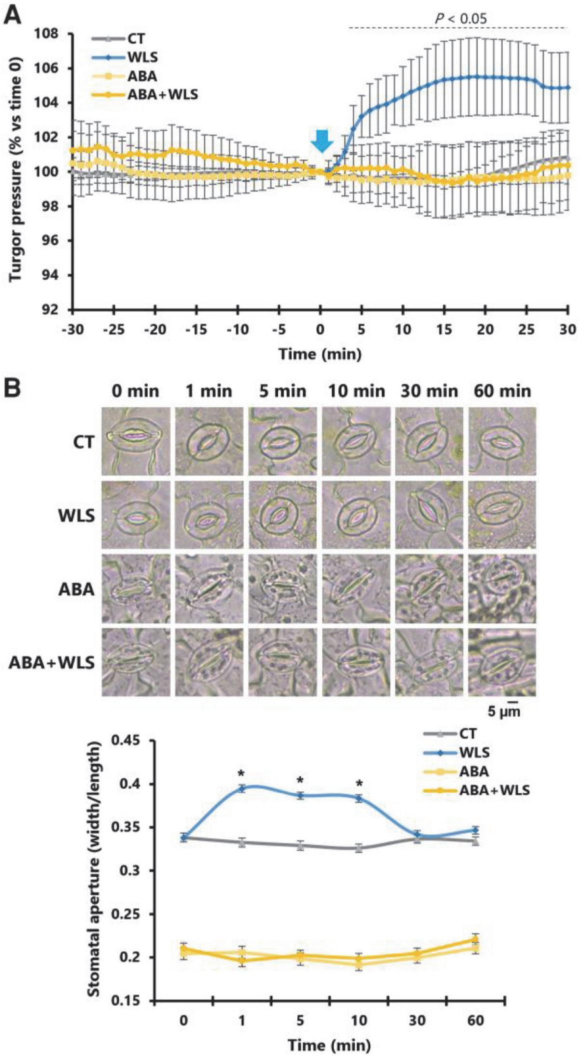

Figure 3. Systemic changes in hydraulic pressure and stomatal responses in WT plants subjected to WLS. **A)** Continuous systemic leaf turgor pressure measurements of WT plants from 30 min prior (−30 min) to 30 min post-WLS (30 min) (applied at 0-min time; indicated with an arrow). Graph shows hydraulic pressure in CT plants and plants subjected to WLS. Water (CT) are ABA (50 μM; ABA and ABA + WLS) were applied to plants by spraying 150 min before WLS application. Hydraulic pressure is represented as the percentage of the initial measured turgor pressure at 0 min. Results are presented as the means ± SE. Statistical analysis was performed with a 2-sided Student's *t*-test (*$P < 0.05$; $n = 12$). Asterisks represent significant differences comparing each time point versus time 0 within the same treatment. All experiments were repeated at least 5 times with 3 plants per treatment. **B)** Systemic stomatal aperture response of *Arabidopsis* to WLS. Representative images of stomata from CT and plants treated (50 μM; ABA and ABA + WLS) or untreated with ABA (WLS; sprayed with water) 150 min before WLS are shown on left, and line graphs showing stomatal aperture measurements at 1, 5, 10, 30, and 60 min following WLS application are shown on right. Results were obtained using at least 20 different plants for each time and treatment (means ± SE, $n = 500$). Stomatal aperture data were compared with CT plants at each time point using 2-sided Student's *t* test (*$P < 0.05$). Scale bar in **B)** represents 5 μm. CT, control.

Liu et al. 2017; Bui et al. 2020; Yang et al. 2022). However, most of these studies did not focus on rapid transcriptomic responses and/or did not use soil or peat for plant growth. To examine the systemic response of shoots from peat soil-grown plants subjected to WLS, we conducted a transcriptomic analysis of WT plants subjected to 0-, 10-, 30-, and 60-min WLS (Fig. 6; Table 1; Supplemental Tables S1 to S9). In addition, we tested the expression of several known hypoxia- and ROS-related transcripts by reverse transcription quantitative PCR (RT-qPCR) at 0- and 60-min post-WLS initiation, to ascertain that the waterlogging treatment we applied to plants induced systemic responses to hypoxic conditions (Supplemental Fig. S2). WLS caused the altered expression of over 2,400, 3,500, and 6,300 transcripts within 10-, 30-, and 60-min stress initiation respectively, with over 300, 800, and 3,800 transcripts uniquely altered in each of these time points (Fig. 6A; Supplemental Tables S1 to S6). Collectively, transcripts altered in systemic tissues in response to WLS contained a high representation of stress-, stimuli-, and anoxia-response transcripts, as well as transcripts involved in hormone, cell communication, and biotic and abiotic responses (Fig. 6B; Supplemental Table S7). A substantial overlap was found between the transcripts identified by our study in systemic tissues of plants subjected to WLS (Fig. 6A) and transcripts identified by several other studies (Bui et al. 2020; Tamura and Bono 2022) in whole plants subjected to hypoxia or submergence (Fig. 6C; Supplemental Table S8). Less overlap was nevertheless found with transcriptomic data obtained from shoots of plants subjected to anoxic conditions or a simulated WLS (Tsai et al. 2014; van Veen et al. 2016), potentially due to the different conditions and time points used in the 2 studies (Fig. 6D; Supplemental Table S8).

When comparing the transcripts significantly altered in our data set (Fig. 6A) with different sets of transcripts significantly altered in plants subjected to different stresses, hormone treatments, or ROS (Zandalinas et al. 2019; Zandalinas, Sengupta, et al. 2021), it was found that many cold-, hypoxia-, wounding-, and ozone-response transcripts are altered in their expression in shoots of plants subjected to WLS (Tables 1 and S9). In addition, many ROS-response and/or ROS wave–response transcripts, previously identified by other studies (Zandalinas et al. 2019), were altered in their expression in shoots of plants subjected to WLS.

Previous studies identified a core set of 49 hypoxia-response genes (Mustroph et al. 2009; Kosmacz et al 2015). We therefore studied the expression of transcripts encoded by these genes in our data set. As shown in Fig. 6E, the expression of transcripts encoded by 24 out of the 49 hypoxia core genes was significantly altered in systemic tissues (leaves) of plants subjected to a WLS (that resulted in the induction of hypoxia in roots; Fig. 1). Included in this group of 24 transcripts was *RbohD* (Fig. 6E), that was found to be under the control of hypoxia-response ERFVII factors (e.g. RAP 2.12; Yao et al. 2017; Supplemental Tables S1 to S3), and is a key player in the ROS wave response

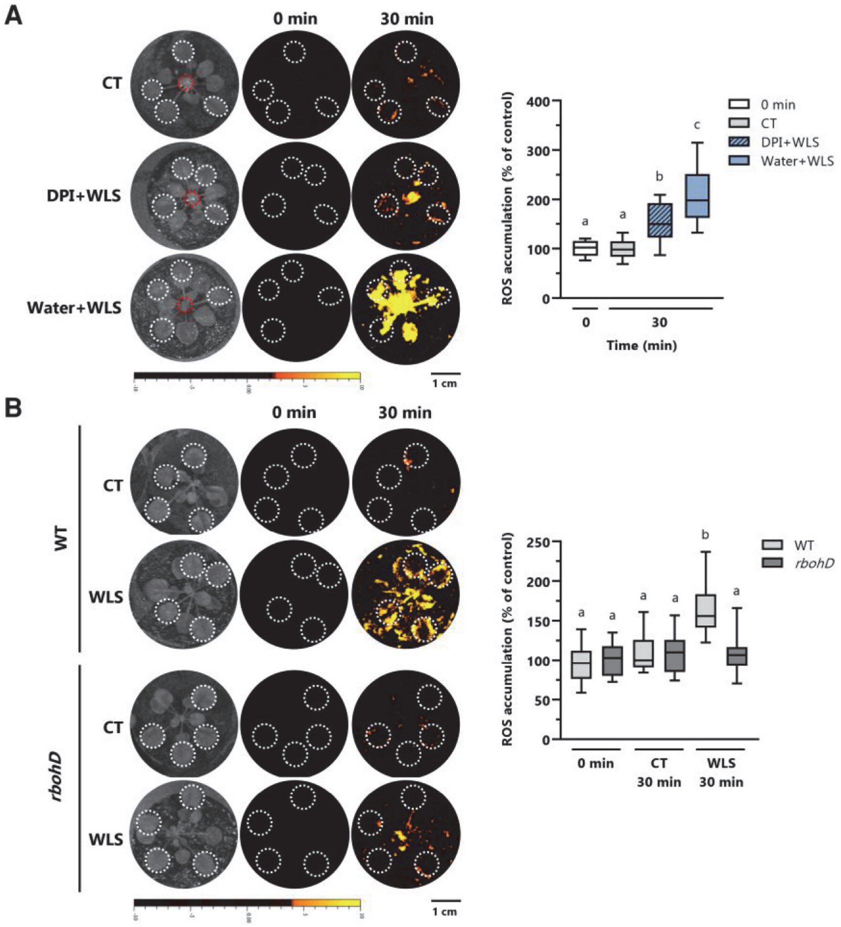

Figure 4. Suppression of the systemic ROS signal triggered by WLS. **A)** The NADPH oxidase inhibitor DPI (50 μM) or water were applied in a drop of agarose to the middle point between the root system and the shoot and plants (indicated with a dashed red circle) and plants were fumigated with H_2DCFDA for 30 min before applying WLS. Representative time-lapse images of whole-plant ROS accumulation in treated and untreated *Arabidopsis* plants are shown alongside box plots of combined data from all plants used for the analysis at the 0- and 30-min time points. **B)** Time-lapse imaging (left) and box plots (right) of combined ROS accumulation data in WT and the *rbohD* mutant in response to a sudden WLS treatment. Radiant efficiency values were measured in the regions of interest indicated by ovals in the images in **A)** and **B)** using the math tools of Living Image 4.7.2 software. Accumulation of ROS was determined by subtracting the signal of the initial time point (0-min) from the time point of interest. Color scale indicates intensity of calculated ROS accumulation. Magnification bars in **A)** and **B)** are 1 cm. Data are shown as box and whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Center line corresponds to the median and the whiskers to the maximum and minimum values. Different letters denote significance at $P < 0.05$ (ANOVA followed by a Tukey's post hoc test). All experiments were repeated at least 3 times with 8 plants per repeat. CT, control; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate.

of plants to WLS (Fig. 4B), as well as other stresses (Fichman et al. 2019; Miller et al. 2009).

To examine what proportion of the systemic transcriptomic response of *Arabidopsis* shoots to WLS is dependent on RBOHD, we conducted an RNA-seq analysis on WT and *rbohD* mutant plants subjected to WLS for 0 and 60 min (Supplemental Tables S10 to S14). As shown in Fig. 7A, over 1,800 transcripts that were altered in WT plants in response to WLS were not altered in the *rbohD* mutant. In addition, over 3,000 transcripts were specifically altered in the *rbohD* mutant, but not WT (Fig. 7A; Supplemental Table S11). As shown in Table 1 (Supplemental Table S12), over 20% of all stress-, ROS-, hypoxia-, and hormone-response transcripts were altered in their expression in the combined group of RBOHD-dependent transcripts (1,806 + 3,028). RBOHD-dependent transcripts were also enriched in abiotic-, biotic-, ethylene-, and ABA-response transcripts, and transcripts involved in organic and metabolic processes (Fig. 7B; Supplemental Table S13). In addition, as shown in Fig. 7C, 27%, 29%, and 28% of all transcripts altered in their expression at 10, 30, and 60 min, respectively, were RBOHD dependent (Supplemental Table S14).

Pretreatment with WLS enhances the tolerance of *Arabidopsis* plants to a subsequent submergence stress

The detection of hypoxia-response genes in leaves of plants subjected to WLS (Figs. 6 and 7; Supplemental Fig. S2)

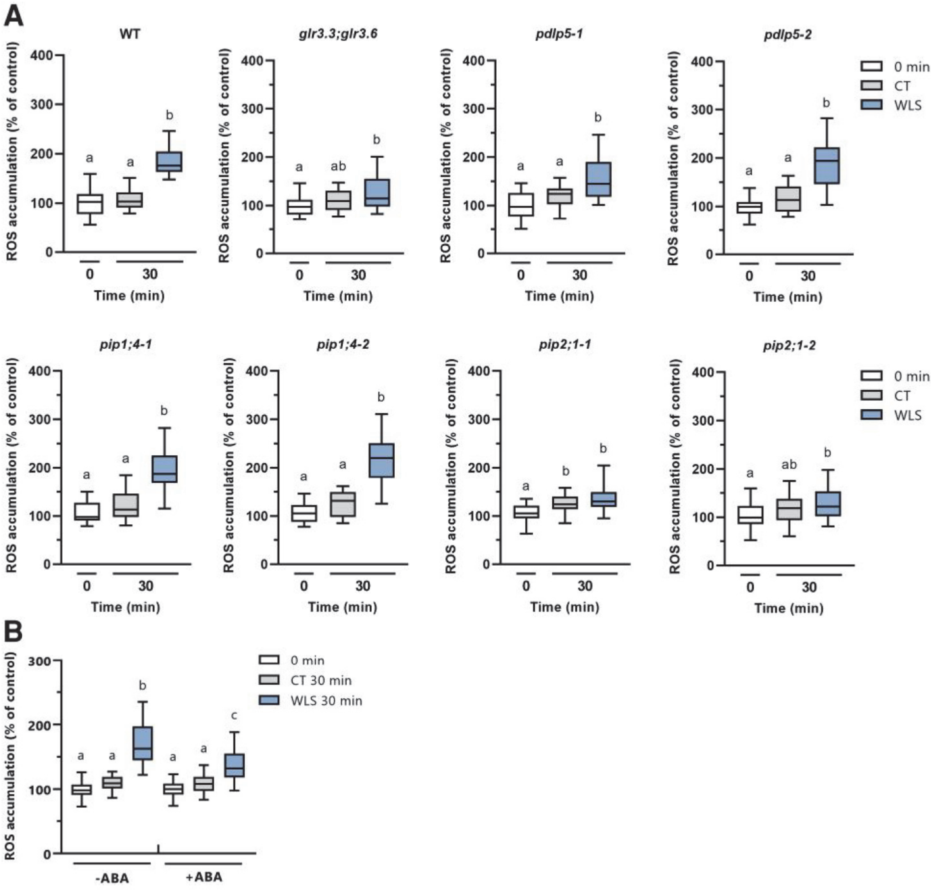

Figure 5. GLR3.3 and GLR3.6 and PIP2;1 are required for WLS-induced rapid systemic ROS signaling in *Arabidopsis*. **A)** ROS accumulation was measured in WT and the *glr3.3;glr3.6*, *pdlp5-1*, *pdlp5-2*, *pip1;4-1*, *pip1;4-2*, *pip2;1-2*, and *pip1;1-2* mutants in response to a sudden WLS treatment. **B)** The effect of pretreatment with ABA on the systemic ROS wave response of WT plants to WLS. Box plots of combined data from all plants used for the analysis at the 0- and 30-min time points are shown. All experiments were repeated at least 3 times with 8 plants per repeat. Representative data are shown as box and whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Center line corresponds to the median and the whiskers to the maximum and minimum values. Different letters denote significance at $P < 0.05$ (ANOVA followed by a Tukey's post hoc test). CT, control; glr, glutamate receptor-like; pdlp5, plasmodesmata localized protein 5; pip1, plasma membrane intrinsic protein 1; pip2, plasma membrane intrinsic protein 2.

prompted us to test whether a pre-treatment with WLS could induce a heightened state of systemic acclimation (i.e. systemic acquired acclimation [SAA]) to a subsequent submergence stress. For this purpose, we treated (acclimated) or untreated (nonacclimated) plants with WLS for 60 min and then submerged them under water for 14 d (water submergence stress). We then allowed plants to recover for 14 more d and scored for leaf injury index (Fig. 8A). As shown in Fig. 8B, compared to nonacclimated/treated plants, WLS-treated/acclimated plants displayed a significantly decreased level of leaf injury. This finding suggested that pretreatment with WLS can prime plants to be more tolerant to a subsequent submergence stress treatment.

Discussion

Plants can respond to stress within seconds to minutes of stress initiation triggering multiple molecular, metabolic, and physiological mechanisms (Suzuki et al. 2015; Choudhury et al. 2018; Devireddy et al. 2018; Kollist et al. 2019). Rapid responses to stress are especially important for plants experiencing the initiation of WLS in the field or in nature that could potentially be followed by partial or complete submergence if the advance of flood water or downpour will not stop (Voesenek and Bailey-Serres 2015; Loreti et al. 2016; Pucciariello and Perata 2017; Sasidharan et al. 2018, 2021; Fig. 8C). Here, we report that a sudden WLS treatment of *Arabidopsis* roots is followed by rapid changes in the expression of many hypoxia-response transcripts in shoots in a time-dependent manner (Fig. 6; Table 1) and that these changes are associated with enhanced tolerance of plants to a subsequent submergence stress treatment (Fig. 8). These findings reveal that plants rapidly activate hypoxia acclimation pathways in shoots (while still under aerobic conditions) potentially in anticipation of an impending partial or complete submergence (that would be accompanied by hypoxia stress; Fig. 8C). This

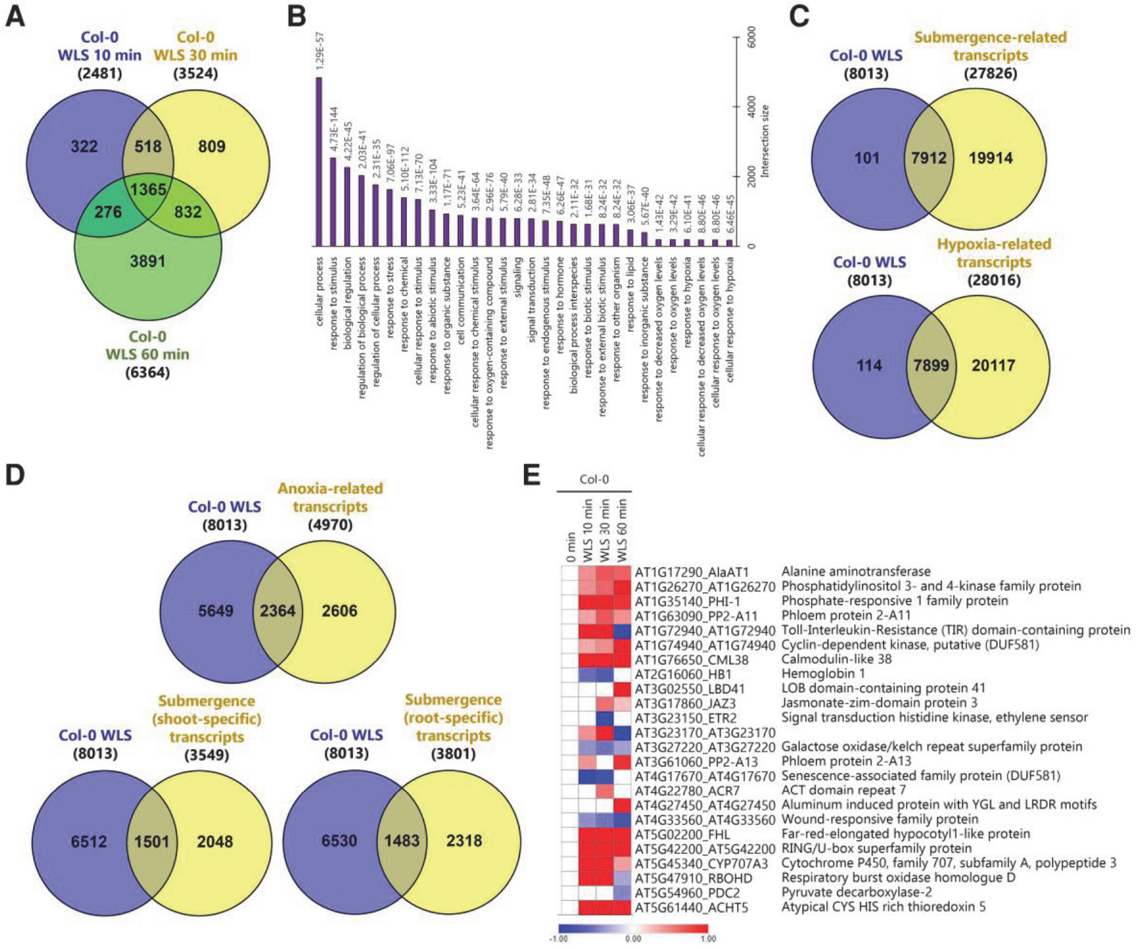

Figure 6. Transcriptomic analysis of the systemic response of WT and *rbohD* plants subjected to WLS. **A)** Venn diagram showing the overlap in transcripts significantly altered in WT after 10, 30, and 60 min of WLS compared to 0 min. **B)** The 30 most statistically significant categories found in biological process (BP) from gene ontology (GO) annotation of all the transcripts (8,013) altered in systemic tissues in response to WLS in WT. **C) and D)** Comparison between the transcripts altered in WT in response to WLS in this study (8,013) and transcriptomic data from previous studies. References used for the comparisons in **C)** are Bui et al. (2020) (submergence-related transcripts) and Tamura and Bono (2022) (hypoxia-related transcripts). In **D)**, the references used are Tsai et al. (2014) (anoxia-related transcripts) and van Veen et al. (2016) (shoot- and root-specific transcripts). **E)** Heatmap of hypoxia core gene (Mustroph et al. 2009) expression (24 out of 49 that were significantly altered in leaves following the WLS treatment).

response could be analogous to other rapid systemic responses, e.g. to pathogens, insects, and/or abiotic stresses such as excess light or temperature extremes (Kollist et al. 2019), and represents an important evolutionary advantage for multicellular organisms that are sessile.

In our study, we used an abrupt WLS treatment that caused mild hypoxic conditions to the root system (Fig. 1). In addition, this treatment generated a hydraulic wave (Fig. 3), most likely a result of the rapid changes in water pressure around the root system caused by the WLS treatment. The changes in water pressure and the initiation of hypoxia conditions at the roots could have caused the accumulation of ROS in the mitochondria and/or at the apoplast (by RBOHs and other oxidases) of root cells that triggered the ROS wave (Fig. 2). In addition, changes in water pressure around the root system could have caused the triggering of the calcium wave in the roots potentially due to the release of glutamate or other factors into the xylem/vascular system (e.g. Gao et al. 2023; Grenzi et al. 2023) or as a response to oxygen deprivation (e.g. Yang et al. 2022). We observed 3 different systemic waves (ROS, calcium, and hydraulic; Figs. 2 and 3) that propagated from the root system to the shoot (leaves) and were associated with a systemic (leaf) transcriptomic response that included a mixture of stress-response transcripts (some directly related to hypoxia and some not; Table 1). The expression of some of these systemic transcripts was dependent on RBOHD (Fig. 7), suggesting that at least part of the systemic response was dependent on the ROS wave. Importantly, we found that the WLS treatment primed plants to be more tolerant to a subsequent (complete) submergence of plants (Fig. 8), potentially a result of the systemic (leaf) hypoxia response induced by the (root) WLS treatment (Figs. 6 and 7).

Table 1. Representation of stress-, hormone-, and ROS-response transcripts in the different groups of transcripts significantly altered in WT and *rbohD* (RBOHD-dependent transcripts) plants in response to WLS

Stress/ treatment	WT WLS 10 min (2,481)	WT WLS 30 min (3,524)	WT WLS 60 min (6,364)	WT WLS 10 + 30 + 60 min (8,013)	RBOHD-dep (1,806 + 3,028)
Cold (622)	49.0	**59.5**	**63.7**	**76.4**	24.0
Hypoxia (358)	40.8	47.2	**51.1**	**61.7**	20.1
Drought (3,382)	16.9	25.3	39.1	49.6	26.5
Heat (1,716)	19.6	25.3	36.2	46.4	24.7
Salt (1,614)	26.1	34.9	35.5	48.6	25.9
Wounding (3,882)	22.6	32.8	44.3	**56.0**	24.6
Ozone (764)	29.7	39.8	47.0	**58.9**	27.4
Pathogen (581)	23.1	31.2	35.3	47.0	27.9
Photosynthesis (67)	25.4	34.3	23.9	**52.2**	37.3
TFs (524)	6.3	7.1	8.4	11.3	6.5
ROS responsive (1,282)	37.8	48.8	**52.7**	**67.4**	25.0
O_2^- (286)	37.4	49.0	46.2	**62.9**	29.4
$^1O_2$ (297)	33.0	47.8	44.1	**62.6**	25.3
H_2O_2 (956)	43.2	**52.6**	**58.5**	**72.1**	23.7
ROS wave (82)	**63.4**	**74.4**	**67.1**	**84.1**	26.8
Hypoxia core (49)	36.7	40.8	40.8	49.0	14.3
ET (255)	13.3	17.3	38.0	43.1	27.5
ABA (1,460)	19.7	28.9	45.9	**56.6**	27.3
BL (276)	37.7	49.3	**56.5**	**70.3**	25.7
CK (335)	20.0	23	44.8	**52.8**	24.8
GA (43)	41.9	46.5	39.5	**60.5**	30.2
IAA (436)	30.7	39.9	48.4	**59.4**	31.2
JA (3,877)	21.2	30.8	44.4	**55.8**	26.4
SA (217)	21.7	34.6	**61.3**	**68.2**	22.1

The transcripts significantly altered ($P < 0.05$, negative binomial Wald test followed by Benjamini–Hochberg correction) in plants in response to stress, hormone, and ROS (left column) from categories “cold,” “drought,” “heat,” “salt,” “wounding,” “ozone,” “pathogen,” “photosynthesis,’ “TFs,’ “ROS responsive,” “O_2^-,” “$^1O_2$,” “H_2O_2,” “ROS wave,” “ABA,” “BL,” “CK,” “GA,” “IAA,” “JA,” and “SA” were obtained from Zandalinas et al. (2019) and Zandalinas, Sengupta, et al. (2021); the transcripts included in “hypoxia core” were taken from Mustroph et al. (2009); and the transcripts in “hypoxia” and “ET” categories were obtained from the GO database using as filter the term IDs GO:0071456, GO:0036293, GO:0036294, and GO:0001666 and for "hypoxia", and GO:0009723, GO:0009873, and GO:0071369 for "ET". The percent of transcripts included in each of these sets (stress-, hormone-, and ROS-response transcripts), found in the data sets obtained in the current study (top row), is shown. The total number of transcripts included in each of the stress-, hormone-, and ROS-response transcript data sets is shown in brackets (left column). Values above 50% are highlighted in bold.

ABA, abscisic acid; ET, ethylene; BL, brassinolide; CK, cytokinins; GA, gibberellic acid; IAA, indole-3-acetic acid; JA, jasmonic acid; RBOHD, NADPH/respiratory burst oxidase protein D; RBOHD-dep, RBOHD-dependent transcripts; SA, salicylic acid; TFs, transcription factors; WLS, waterlogging stress; WT, wild type.

Previous studies conducted with trees and other plants, including crop plants, identified stomatal responses, changes in hydraulic pressure (in the roots and stems), and changes in transcriptomic responses, as playing an important role in plant responses and acclimation to WLS (e.g. Sellin 2001; Rodríguez-Gamir et al. 2011; Liu et al. 2012; Striker et al. 2014; Gasch et al. 2016; Jurczyk et al. 2016; Martínez-Arias et al. 2020; Repo et al. 2021; Liu et al. 2022). These studies were however conducted in plants subjected to longer WLS periods and did not address rapid responses that occur within minutes of WLS initiation. In our work, we demonstrate that stomatal, hydraulic, and transcriptomic responses occur in systemic tissues of *Arabidopsis* plants within 1 to 10 min of WLS application (Figs. 2, 3, and 6), revealing that many important responses to WLS might occur much earlier than previously thought. The overlap between many of the responses identified by our study and previous studies (stomatal, hydraulic, and transcriptomics) suggests that the early changes identified in our study play an important role in plant acclimation to WLS.

Cellular responses to hypoxia and/or anoxia have been extensively studied, and RBOHD was found to play a key role in ROS accumulation during the initial stages of anoxia stress (as well as in plant acclimation to anoxia; Liu et al. 2017). Our study reveals that in addition to controlling local responses to anoxia (Liu et al. 2017), RBOHD could also be involved in controlling systemic responses to WLS (Figs. 4 and 7; Table 1). The activation of RBOHD and other ROS-generating mechanisms (e.g. mitochondrial; Schmidt et al. 2018), in roots following the sudden WLS treatment, could therefore trigger the ROS wave that propagates in a cell-to-cell fashion from the roots all the way to the shoots and, together with the other systemic signals triggered by WLS (Figs. 2 and 3), activates the expression of many different hypoxia acclimation mechanisms in the shoots (Figs. 2, 4, 6, and 8; Table 1). Our findings that the application of a drop of DPI to the middle point between the roots and the shoots suppresses the WLS-induced ROS wave in shoots (Fig. 4A) strongly support this possibility. As with previous studies of rapid systemic ROS signaling in *Arabidopsis* in response to different abiotic stresses, the WLS-triggered ROS wave required the function of GLR3.3 and GLR3.6 and PIP2;1 (Fig. 5A; Fichman et al. 2021; Fichman and Mittler 2021). In contrast, PDLP5 that was previously found to be required for systemic responses to wounding or excess light stress (Fichman et al. 2021; Fichman and Mittler 2021) appeared to not be required for this response (Fig. 5A). These findings suggest that the ROS wave induced by WLS (Fig. 2A) is mediated via a somewhat different molecular mechanism than that triggered in response to excess light or wounding in *Arabidopsis* (Fig. 5A; Fichman et al. 2021; Fichman and Mittler 2021). Further studies are required to address the molecular mechanisms regulating the ROS wave response to WLS.

We previously reported that the calcium, ROS, and hydraulic waves are integrated in *Arabidopsis* during systemic responses to wounding (applied to a single leaf) and require the function of GLR3.3 and GLR3.6 (Fichman et al. 2021; Fichman and Mittler 2021). Although under WLS, the ROS wave was dependent on GLR3.3 and GLR3.6 function (Fig. 5A), it is not clear whether the calcium and hydraulic waves are also dependent on these calcium-permeable

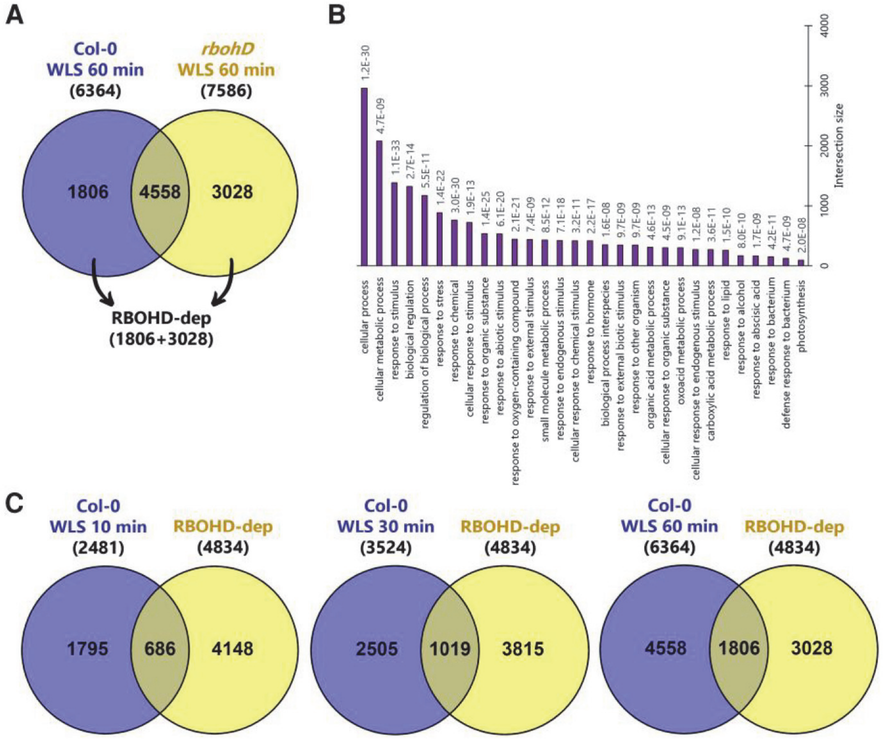

Figure 7. RBOHD-dependent systemic transcriptomic responses to WLS. **A)** Overlap between transcripts significantly altered in WT and *rbohD* plants following 60 min of WLS treatment. **B)** The 30 most statistically significant categories found in biological process (BP) of the GO annotation for the RBOHD-dependent transcripts (1,806 + 3,028) at time 60 min. **C)** Venn diagrams showing the overlap between the RBOHD-dependent transcripts at 60 min (1,806 + 3,028) and the transcripts altered in WT after 10, 30, and 60 min of WLS. RBOHD-dep, RBOHD-dependent transcripts.

channels. In addition, the function of CAX1, previously found to control calcium and ROS signaling under submergence and anoxia stresses (Yang et al. 2022), in mediating the ROS wave during WLS is unclear. One possibility is that in roots, under waterlogging-mediated anoxia stress, CAX1 is required for ROS production by RBOHD (Yang et al. 2022), while in shoots, under aerobic conditions, GLR3.3 and GLR3.6 are required for ROS production by RBOHD (Fig. 5A; Fichman et al. 2021; Fichman and Mittler 2021). As calcium signaling plays such a key role in triggering abiotic and biotic responses in plants (e.g. Luan and Wang 2021), further studies are needed to address the potential contribution of these calcium signaling mechanisms, as well as others, to the overall systemic response of plants to WLS.

Hydraulic waves are thought to play an important role in the systemic response of plants to different stresses (Kloth and Dicke 2022; Grenzi et al. 2023). The changes in water pressure propagating through the vascular system of plants were proposed to trigger mechanosensory proteins that translate the hydraulic signal into a calcium signal and subsequently a ROS accumulation response in systemic tissues (Gilroy et al. 2016; Kloth and Dicke 2022). As the systemic response of *Arabidopsis* to WLS involves a rapid systemic hydraulic response (Fig. 3A), that was followed by systemic calcium and ROS responses (Fig. 2), it is possible that these 3 waves are interlinked in *Arabidopsis* during WLS. This possibility is also supported by the finding that pretreatment of plants with ABA, that suppressed the hydraulic wave (Fig. 3A), also suppressed the ROS wave (Fig. 5B). The association between the hydraulic, calcium, and ROS waves should be addressed in future studies using experimental systems like the one presented in this study, using for example mechanosensory mutants such as the MECHANOSENSITIVE ION CHANNEL LIKE 2 or 3 (*msl2* or *msl3*) mutants, previously found to be required for the propagation of the ROS wave (Fichman et al. 2021, 2022). The association between the stomatal opening response, ABA, and the hydraulic wave (Fig. 3) should also be pursued in future studies as it suggests that the degree of stomatal aperture openness at the time WLS occurs might impact hydraulic waves and other systemic responses to this stress. In this respect, it should be mentioned that hydraulic waves were recently proposed to control glutamic acid release from cells during systemic responses to wounding, controlling the systemic calcium wave (Grenzi et al. 2023), as well as linked to the mobilization of glucohydrolases, which are important for the regulation of electrical signals during wound responses (Gao et al. 2023). Hydraulic waves might therefore play a central role in regulating systemic responses in plants, and elucidating their function requires further studies.

The potential dependence of systemic hydraulic responses to WLS on stomatal aperture (Fig. 3) could play an important role during conditions of stress combination (Zandalinas and Mittler 2022). It was previously shown that in combination

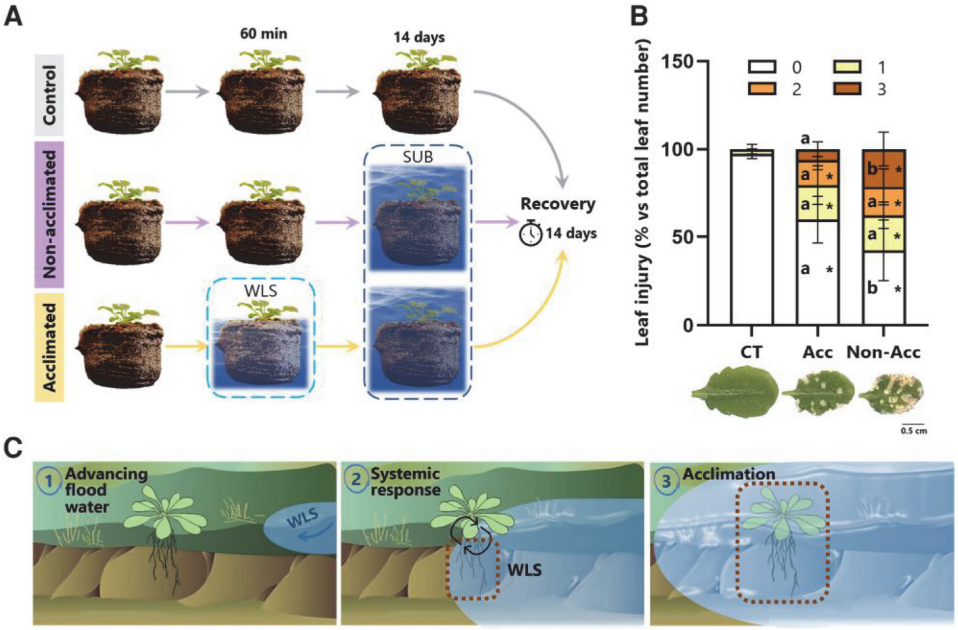

Figure 8. Pretreatment of WLS protects plants from a potential submergence stress. **A)** The experimental design used. Plants were either untreated (Non-Acc) or pretreated (Acc) with WLS for 60 min and then completely submerged for 14 d. The leaf injury of CT (nonpretreated or submerged), Acc, and Non-Acc plants was then scored at 14-d postrecovery from complete submergence. **B)** Representative images of leaves from CT, Acc, and Non-Acc plants and a bar graph of leaf injury scored from the different plants. Leaf injury index was scored as noninjury (Level 0), low level injury (Level 1), medium level injury (Level 2), and high level injury (Level 3). All experiments were repeated at least 3 times with 6 plants per repeat. Statistical significance was determined using a 1-way ANOVA followed by Tukey's post hoc test ($P < 0.05$). Results are presented as the means ± SE. * Denotes significant differences compared to CT treatment, and letters denote significant differences between Acc and Non-Acc plants within the same injury level. **C)** A model for the proposed function of the plant WLS systemic response. Advancing flood water is shown in Panels 1 and 2 to cause WLS and induce a systemic response in leaves. The systemic response of plants to WLS (that involves multiple hypoxia/anoxia genes) is then shown in Panel 3 to cause plant acclimation that protects plants from a potential subsequent submergence event that could occur if the flood water will not stop advancing. As the advance of flood water can be fast, the systemic response of plants to WLS needs to be rapid. Acc, acclimated; CT, control; Non-Acc, nonacclimated.

with other stresses, such as heat stress (that is becoming a major problem worldwide due to global warming; Bailey-Serres et al. 2019; Masson-Delmotte et al. 2021; Zandalinas, Fritschi, and Mittler 2021), WLS could become substantially more lethal to some crops (e.g. Lin, Lin, et al. 2015; Lin and Chen 2015; Zhen et al. 2020; Shao et al. 2022), highlighting the importance of studying plant responses to complex conditions of stress combination (Zandalinas, Fritschi, and Mittler 2021; Zandalinas and Mittler 2022). As rapid systemic responses to different stresses could be conflicting during stress combination (Zandalinas, Fichman, Devireddy, et al. 2020), more studies are needed to address the effects of heat and other stresses on the rapid systemic responses of plants to WLS. This is especially important as WLS causes rapid alterations in stomatal aperture (Fig. 3B), and stomatal responses to different stresses, occurring during stress combination, were shown to be contradicting (Zandalinas and Mittler 2022).

Although the 10-min WLS treatment described in the current study resulted in a decrease in oxygen levels around the plant root system (Fig. 1B), and the systemic transcriptomic response at 10 min included hypoxia-related transcripts (Fig. 6; Table 1), the rapid systemic transcriptomic response of *Arabidopsis* to the sudden WLS treatment observed in our study might in fact reflect a combination of different systemic signals, some to hypoxia conditions (Fig. 1B), some to changes in hydraulic pressure (Fig. 3A), and some to increase in ethylene levels around the root system (Hsu et al. 2011; Tsai et al. 2014). One way to dissect the different causes of the observed rapid systemic transcriptomic response could be to study it in mutants deficient in hypoxia responses (e.g. mutants impaired in GROUP VII ETHYLENE RESPONSE FACTORs; Gasch et al. 2016), mutants deficient in ethylene signaling (Myers et al. 2023), and mutants deficient in hydraulic wave responses (Fichman and Mittler 2021). Such studies could distinguish between hypoxia-, ethylene-, or hydraulic-driven transcriptomic responses. In this respect, it is important to note that in contrast to the large number of hypoxia-response transcripts accumulating at 10 min following WLS initiation, the number of ethylene-response transcripts accumulating at 10 min was not high (Table 1). This finding, together with the oxygen level measurements around the root system at 10 min (Fig. 1B), suggests that the systemic hypoxia-related response observed in our study at 10 min is indeed at least partially linked to the induction of hypoxic conditions at the root system following WLS.

Taken together, our study suggests that a sudden treatment of WLS (that could occur for example when flood waters advance during a flooding event; Fig. 8C) results in the activation of multiple rapid systemic signals (ROS, calcium, and hydraulic; Figs. 2 and 3) and the rapid induction of hypoxia response and acclimation mechanisms in systemic tissues (Figs. 6 and 7). In addition, we show that such treatment can induce in plants a state of SAA to a subsequent submergence stress (Fig. 8B) that could occur if the flooding waters keep advancing and completely cover the plant (Fig. 8C).

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis* (*A. thaliana*) Col-0 and the mutants *rbohD* (Torres et al. 2002), *glr3.3glr3.6* (Mousavi et al. 2013; Nguyen et al. 2018), *pdlp5* (2 independent alleles; Fichman et al. 2021), *pip2;1* (2 independent alleles; Rodrigues et al. 2017; Fichman et al. 2021), and *pip1;4* (2 independent alleles, Fichman et al. 2021) were germinated and grown on peat pellets (Jiffy-7; Jiffy International, Kristiansand, Norway), under controlled conditions of 10-h/14-h light/dark regime, 50 μmol photons s^{-1} m^{-2}, and 21 °C for 4 wk.

WLS application

To induce WLS, we grew plants as described above for 4 wk and allowed the water content of the peat pellets to reach 54 ± 2% of complete peat soil water saturation (each peat pellet was first weighed when it was fully saturated with water and then weighed regularly until 54 ± 2% of fully saturated weight was achieved). Plants were then treated or untreated with the different dyes for imaging as described below, placed in a tray that allowed watering to saturation inside the imager, or back under the growth light, as described below, rapidly watered until the water levels reached all the way to the top of the peat soil (97 ± 5% water capacity measured 60 min following watering), and subjected to imaging or other analyses as described below (Fig. 1A). Oxygen levels around the root system were measured before and during the WLS with the Bante821 portable dissolved oxygen meter (Bante Instruments, Shanghai, China).

Whole-plant imaging of ROS and calcium levels

As previously described (Fichman et al. 2019; Zandalinas, Fichman, Devireddy, et al. 2020; Zandalinas, Fichman, and Mittler 2020; Fichman, Zandalinas, et al. 2020; Fichman and Mittler 2021), plants were fumigated for 30 min with 50 μM H_2DCFDA (Millipore-Sigma, St. Louis, MO, USA) for ROS imaging or with 4.5 μM Fluo-4-AM (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for calcium imaging, using a nebulizer (Punasi Direct, Hong Kong, China) in a glass container. To inhibit ROS propagation, DPI (50 μM, Millipore-Sigma, St. Louis, MO, USA) and water were applied in a drop of 0.3% (w/v) agarose to the midpoint of the rosette that connects the shoot to the root system 30 min before WLS, as described by Fichman et al. (2019). Plants were also fumigated with a solution containing DPI at a final concentration of 50 μM together with the dye H_2DCFDA as described above. Following fumigation, plants were subjected to a sudden WLS as described above, a black plastic mask with holes was placed above the tray containing the water to avoid the background of water autofluorescence, and imaging using the IVIS Lumina S5 fluorescence imager (PerkinElmer, Waltham, MA, USA). Fluorescence images (excitation/emission 480/520 nm) were acquired every minute for 60 min as described by Fichman et al. (2019). Images were analyzed with Living Image 4.7.2 software (PerkinElmer), and data analysis was performed by measuring radiant efficiency in regions of interest as explained previously (Fichman et al. 2019). Accumulation of ROS or calcium was compared to the 0-min time point and determined by subtracting the fluorescent signal of the initial time point (0-min) from the time point of interest. The 0-min time point was the initial image, which results in no signal being visualized due to fluorescence subtraction. All experiments were repeated at least 3 times each with 8 plants.

H_2O_2 detection

H_2O_2 quantification in systemic leaves was performed using Amplex-Red (10-acetyl-3,7-dihydroxyphenoxazine [ADHP]; Thermo Fisher Scientific, Waltham, MA, USA) as described by Fichman et al. (2022). Systemic leaves from control and WLS-treated plants were immediately frozen and ground to fine powder, resuspended in 50-μL 0.1 M TCA (Thermo Fisher Scientific, Waltham, MA, USA), and centrifuged for 15 min at 12,000 × g, 4 °C. The supernatant was buffered with 1 M phosphate buffer pH 7.4, and the pellet was dried and used for dry weight calculation. H_2O_2 quantification in the supernatant was performed according to the MyQubit-Amplex-Red Peroxide Assay manual (Thermo Fisher Scientific, Waltham, MA, USA), using a calibration curve of H_2O_2 (Thermo Fisher Scientific, Waltham, MA, USA) as described by Fichman et al. (2022).

Hydraulic pressure measurements

Changes in systemic leaf turgor pressure following WLS were recorded using the ZIM-probe system (Yara International ASA, Oslo, Norway; Zimmermann et al. 2013), as described by Fichman and Mittler (2021). Briefly, a single leaf of 4-wk-old plants was connected to 2 magnetic probes that included a pressure sensor between them (Zimmermann et al. 2013). The turgor pressure force against the magnetic pressure was recorded and transmitted to a receiver every minute. ABA treatment was performed by spraying ABA (50 μM; Millipore-Sigma, St. Louis, MO, USA) on the entire rosette 30 min prior to connecting leaves to the magnetic probes. Control plants were simultaneously sprayed with distilled water. Following magnetic probe attachment, the system was allowed to stabilize for 2 h and plants were subjected to the WLS treatment. Pressure values were recorded for an additional 60 min following the stress application. Hydraulic pressure was calculated as the percentage of the initial measured turgor pressure at 0 min, which is the pressure in the leaf right before the stress application. All experiments were performed between 9 AM and 1 PM. Each data set includes average and SE of 6 to 12 biological repeats.

Measurements of stomatal aperture

A thin layer of nail polish (450B clear nail protector-Wet N Wild; Markwins Beauty Products, CA, USA) was applied to the abaxial surface of the leaf avoiding the major veins. The nail polish was left to dry for approximately 10 min and then peeled off with tweezers. Impressions were mounted pointing upward with double-sided tape (Scotch) on a microscope slide. Stomata images were captured with an EVOS XL microscope (Invitrogen by Thermo Fisher Scientific, Waltham, MA, USA). Both width and length of stomatal aperture were measured using ImageJ (https://imagej.nih.gov/ij). Stomatal aperture was calculated as a ratio of stomatal pore width to stomatal pore length as described by Wang et al. (2019). All experiments were conducted between 9 AM and 1 PM. Results include stomatal aperture values from at least 20 different plants for each time point and treatment ($n = 500$).

RNA isolation and transcript expression analysis

Four-week-old WT and *rbohD* plants were subjected to WLS, and systemic leaves (leaves number 4, 5, 6, and 7 at 10-leaf rosette stage; Supplemental Fig. S3) were collected and immediately frozen in liquid nitrogen at times 0, 10, 30, and 60 min. Sixty leaves were pooled from 15 different plants for each biological repeat (3 biological repeats were used for each time point and genotype). RNA was extracted using Plant RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Total RNA was used for cDNA synthesis (PrimeScript RT Reagent Kit; Takara Bio, Takara Bio, Kusatsu, Japan). Transcript expression was quantified by RT-qPCR using iQ SYBR Green super mix (Bio-Rad Laboratories, Hercules, CA, USA), as previously described (Fichman et al. 2021), with specific primers for the following transcripts: *ZAT10* (AT1G27730), 5′-ACTAGCCACGTTAGCAGTAGC-3′ and 5′-GTTGAAGTTTGACCGGAAGTC-3′; *RAP2.3* (AT3G16770) 5′-AGCAGATCCGTGGTGATAAAG-3′ and 5′-TATACTCCTC CGCCGTCA-3′; and *ADH1* (AT1G77120), 5′-GATCAT GTGTTGCCGATCTTTAC-3′ and 5′- CTTCTCAGGATCAAC ACCGAG −3′; *HRE2* (AT2G47520), 5′-GGGAAACGAG AGAGGAAGAATC-3′ and 5′-AAAGGTGTACGTGTCTGG C-3′. *Elongation factor 1 alpha* (5′-GAGCCCAAGTTTTTGA AGA-3′ and 5′-TAAACTGTTCTTCCAAGC TCCA-3′) was used for normalization of relative transcript levels. Results, expressed in relative quantity ($2^{-\Delta\Delta CT}$), were obtained by normalizing relative transcript expression (ΔC_T) and comparing it to control WT from local leaf ($\Delta\Delta C_T$). The data represent 15 biological repeats and 3 technical repeats for each reaction. SE and Student's *t* test were calculated with Microsoft Excel.

RNA sequencing and data analysis

RNA libraries were prepared using standard Illumina protocols, and RNA sequencing was performed using NovaSeq 6000 PE150 by Novogene Co. Ltd (https://en.novogene.com/; Sacramento, CA, USA). Quality control for the raw reads was evaluated by FastQC v.0.11.9 (Andrews 2010) and aggregated with MultiQC tool v.1.13.dev0 (Ewels et al. 2016). Adapter content, ambiguous nucleotide, and any sequences with read length less than 20 bp and a Phred score less than 20 were removed from the raw reads with Trim Galore v.0.6.7 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The RNA-seq reads were aligned to the reference genome for *Arabidopsis* (TAIR10) (downloaded from https://ftp.ensemblgenomes.ebi.ac.uk/pub/plants/release-54/fasta/arabidopsis_thaliana/dna_index/), using HISAT2 short-read aligner v.2.2.1 (Kim et al. 2019), which gave a high overall alignment (~98%). Intermediate file processing of sam to sorted bam conversion was conducted using SAMtools v.1.9 (Li et al. 2009). Transcript abundance expressed as fragments per kilobase million (FPKM) was generated using the Cufflinks tool v.2.2.1 from the TUXEDO suite (Trapnell et al. 2012) guided by genome annotation files (downloaded from https://ftp.ensemblgenomes.ebi.ac.uk/pub/plants/release-54/gff3/arabidopsis_thaliana/). Differential gene expression analysis was performed using the Cuffdiff 2 method (Trapnell et al. 2013), also from the same TUXEDO suite. Differentially expressed transcripts were defined as those that had a fold-change with an adjusted $P \leq 0.05$ (negative binomial Wald test followed by Benjamini–Hochberg correction). For the analysis and visualization of the data, R was used with methods and packages available through CRAN (R Core Team 2022, https://www.R-project.org/) or Bioconductor (Huber et al. 2015). Functional annotation and quantification of overrepresented gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were performed using gprofiler2 package v.0.2.1 (Raudvere et al. 2019) using a threshold of ($P < 0.05$). Venn diagrams were created in Venny 2.1 (BioinfoGP, CNB-CSIC). The different stress-, hormone-, and ROS-response transcripts data sets used for comparisons in Table 1 were obtained from Zandalinas et al. (2019) and Zandalinas, Sengupta, et al. (2021), Mustroph et al. 2009 and the GO database using the tool AmiGO (http://amigo.geneontology.org/amigo).

WLS/submergence acclimation assays

For the WLS/submergence acclimation study (Fig. 8A), plants were grown under controlled conditions as described above for 4 wk. Two hours into the photoperiod, plants of uniform size, and developmental stage were divided into 3 groups. One group (acclimated plants) was subjected to WLS stress for 60 min as described above (Fig. 1) and then completely submerged in bins (35 × 25 cm) filled with water to induce submergence stress. The second group (nonacclimated plants) was subjected to the same submergence treatment without a WLS pretreatment. The third group (control plants) remained under controlled conditions throughout the experiment (as described above). Acclimated and nonacclimated plants were maintained under submerged conditions side by side for 2 wk, with leaves completely covered by approximately 10 cm of water. For recovery, plants were removed from the water and allowed to recover under controlled conditions for 2 wk before scoring for leaf injury. To determine leaf damage, 4 different levels of injury were established, according to Balfagón et al. (2019, 2022). Experiments were repeated 3 times consisting of 6 repeats for each condition.

Statistical analysis

All experiments were repeated at least 3 times with 3 biological repeats. Statistical analysis for data presented in Figs. 2, 4, and 5 and Supplemental Fig. S1 was performed using 1-way ANOVA followed by Tukey's post hoc test ($P < 0.05$) in GraphPad. Results are shown as box and whisker plots with borders corresponding to the 25th and 75th percentiles of the data. Different letters denote statistical significance at $P < 0.05$. Statistical analysis for data in Fig. 1 and Supplemental Fig. S2 was performed by 2-sided Student's *t* test (*$P < 0.05$) in Microsoft Excel, and results are presented as means ± SE.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number GSE225407.

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Author contributions

M.A.P.-V. and A.T. performed the experiments and analyzed the data. M.A.P.-V., R.M., T.J., and D.G.M.-C. designed the experiments, analyzed the data, and/or wrote the manuscript.

Supplemental data

Supplemental Fig. S1. The effect of DPI fumigation on systemic ROS accumulation in response to WLS in WT plants.

Supplemental Fig. S2. RT-qPCR analysis of *Zinc finger protein 10* (*ZAT10*), *Ethylene response factor subfamily B-2* (*RAP2.3*), *Alcohol dehydrogenase 1* (*ADH1*), and *Hypoxia responsive ethylene response factor gene 2* (*HRE2*) in systemic leaves of WT plants subjected to WLS.

Supplemental Fig. S3. Position of the leaves used for the transcriptomic analysis shown in Fig. 5.

Supplemental Table S1. Transcripts differentially expressed in WT plants subjected to WLS for 10 min (compared to WT 0 min).

Supplemental Table S2. Transcripts differentially expressed in WT plants subjected to WLS for 30 min (compared to WT 0 min).

Supplemental Table S3. Transcripts differentially expressed in WT plants subjected to WLS for 60 min (compared to WT 0 min).

Supplemental Table S4. List of transcripts uniquely altered in WT at time 10 min.

Supplemental Table S5. List of transcripts uniquely altered in WT at time 30 min.

Supplemental Table S6. List of transcripts uniquely altered in WT at time 60 min.

Supplemental Table S7. Gene ontology and KEGG pathway enrichment of all the transcripts regulated in WT in response to waterlogging (10 + 30 + 60 min) shown in Fig. 6B.

Supplemental Table S8. Comparisons between the list of systemic transcripts regulated in WT in response to waterlogging (8,013) with data sets from previous studies with *Arabidopsis* plants subjected to hypoxia or submergence stress.

Supplemental Table S9. Common transcripts found in the overlapping of the transcriptomic response of WT to WLS with transcripts significantly altered in plants subjected to different stresses, hormone treatments, or ROS (data shown in Table 1).

Supplemental Table S10. Transcripts significantly expressed in *rbohD* plants subjected to WLS for 60 min (compared to *rbohD* 0 min).

Supplemental Table S11. Transcripts found in the comparison between the transcriptional response of WT and *rbohD* plants subjected to WLS for 60 min (Fig. 7A).

Supplemental Table S12. Common transcripts found in the overlapping of the transcriptomic response of *rbohD* to WLS with transcripts significantly altered in plants subjected to different stresses, hormone treatments, or ROS (data shown in Table 1).

Supplemental Table S13. Gene ontology and KEGG pathway enrichment of RBOHD-dependent transcripts (1,806 + 3,028; Fig. 7B).

Supplemental Table S14. Transcripts altered in their expression at 10, 30, and 60 min and that are RBOHD dependent (Fig. 7C).

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Conflict of interest statement. The authors declare no conflict of interest.

Data availability

RNA-seq data were deposited in Gene Expression Omnibus (GEO), under the following accession number: GSE225407.

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