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A systemic whole-plant change in redox levels accompanies the rapid systemic response to wounding

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Letter

Dear Editor,
Reactive oxygen species (ROS) play a central role in the regulation of plant responses to different developmental signals, abiotic stresses, wounding, and pathogen attack (e.g. Fryer et al., 2003; Chang et al., 2004; Mhamdi and Van Breusegem, 2018; Kollist et al., 2019). ROS alter gene expression in response to many of these stimuli through a change in the redox state of different proteins (Mittler, 2017; Huang et al., 2019). In addition to triggering responses at the tissues directly subjected to abiotic stress, ROS regulate rapid whole-plant systemic responses at tissues not directly subjected to the stress, inducing a state of systemic acquired acclimation (SAA), or systemic wound response (SWR; e.g. Miller et al., 2009; Szechyńska-Hebda et al., 2010; Suzuki et al., 2013; Gilroy et al., 2016; Fichman et al., 2019; Zandalinas et al., 2019, 2020a). The ROS-dependent systemic signaling pathway mediating these processes, termed "the ROS wave" (Miller et al., 2009), is regulated by respiratory burst oxidase homolog proteins and mediated through the vascular bundles of plants (Zandalinas et al., 2020b). However, whether the ROS wave that spreads from the local tissues subjected to stress to the rest of the plant triggers alterations in redox levels, is mostly unknown.

Genetically encoded reporters for cellular and glutathione redox changes and their application in plants have led to major advancements in the study of redox and ROS signaling in recent years (e.g. Hanson et al., 2004; Schwarzländer et al., 2008; Meyer and Dick, 2010; Rosenwasser et al., 2010; Beneloujaephajri et al., 2013; Schwarzländer et al., 2016; Exposito-Rodriguez et al., 2017; Lim et al., 2019; Nietzel et al., 2019; García-Quirós et al., 2020; Haber and Rosenwasser, 2020; Ugalde et al., 2020). These reporters have however been primarily used in conjunction with confocal microscopy, limiting their application to the detection of redox changes in specific cells, tissues, and organs (e.g. local responses to wounding; Beneloujaephajri et al., 2013). In contrast, whole-plant detection of redox changes in mature plants grown in soil has been limited. We recently developed a method for live whole-plant imaging of ROS levels in soil-grown plants and used it to study the ROS wave in wild-type plants and different mutants responding to different stimuli (e.g. Devireddy et al., 2020; Fichman et al., 2020; Zandalinas et al., 2020a). Although changes in ROS levels accompanied the systemic response of plants to different stimuli (Fichman et al., 2019), resulting in metabolic and transcriptomic changes that drove SAA or SWR (e.g. Suzuki et al., 2013; Zandalinas et al., 2019, 2020a), it is not known whether a systemic whole-plant redox response also accompanies this rapid signaling process. To test this possibility, we studied cytosolic reduction–oxidation sensitive green fluorescent protein 1 (roGFP1)-expressing plants (Jiang et al., 2006; Meyer et al., 2007; Schwarzländer et al., 2008; Supplementary Methods) subjected to a local injury stimuli (Figures 1, 2). We chose roGFP1 as a tool to measure

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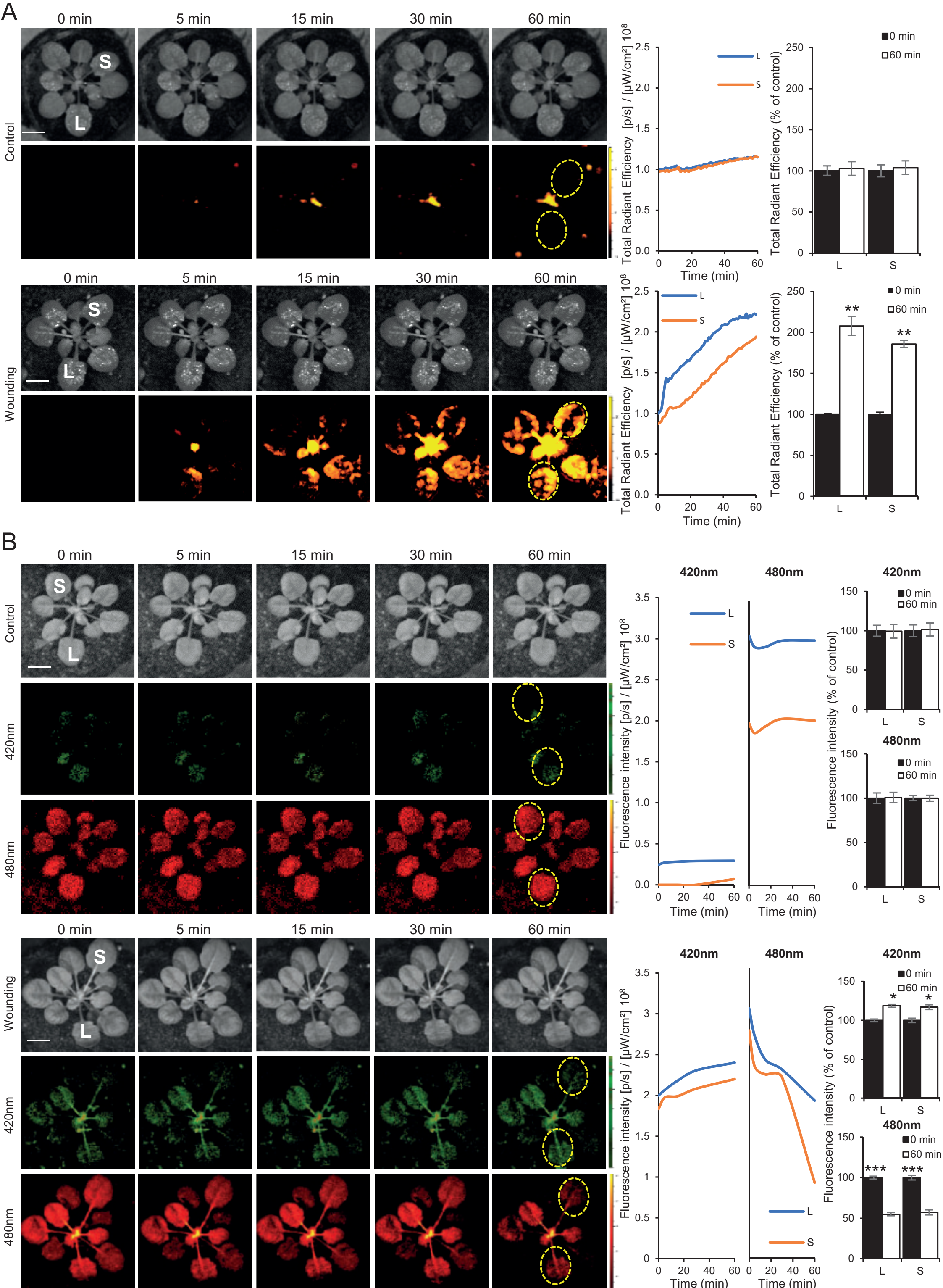

Figure 1 Imaging of ROS and redox levels in whole plants subjected to wounding. A, Left: Representative time-lapse images of whole-plant ROS levels (indicated by DCF oxidation) in wild-type Arabidopsis (Col-0) plants, untreated (control, top), or wounded (bottom; applied to the local leaf only). Middle: Representative line graphs showing continuous measurements of ROS levels in local and systemic leaves over the entire course of the experiment (0–60 min; ROIs used to generate the line graphs are indicated with dashed yellow ovals on left). Right: Statistical analysis of ROS levels in local and systemic leaves at 0 and 60 min. B, Representative time-lapse images, and line and bar graphs, of roGFP1 fluorescence of transgenic plants overexpressing the roGFP1 protein at the cytosol (Jiang et al., 2006), untreated (top), or subjected to wounding applied to leaf L only (bottom; similar to the experimental set up shown in A). roGFP1 fluorescence was measured at excitation/emission (ex/em) of 420/520 nm for oxidized roGFP1 (middle, in green; oxidized), and at ex/em of 480/520 nm (bottom, in red; reduced). Experiments were conducted in three biological repeats, each with two technical repeats. Student's *t* test, SE, $N = 3$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$. Scale bar indicates 1 cm. ex/em, excitation/emission; L, local; S, systemic; and ROI, region of interest.

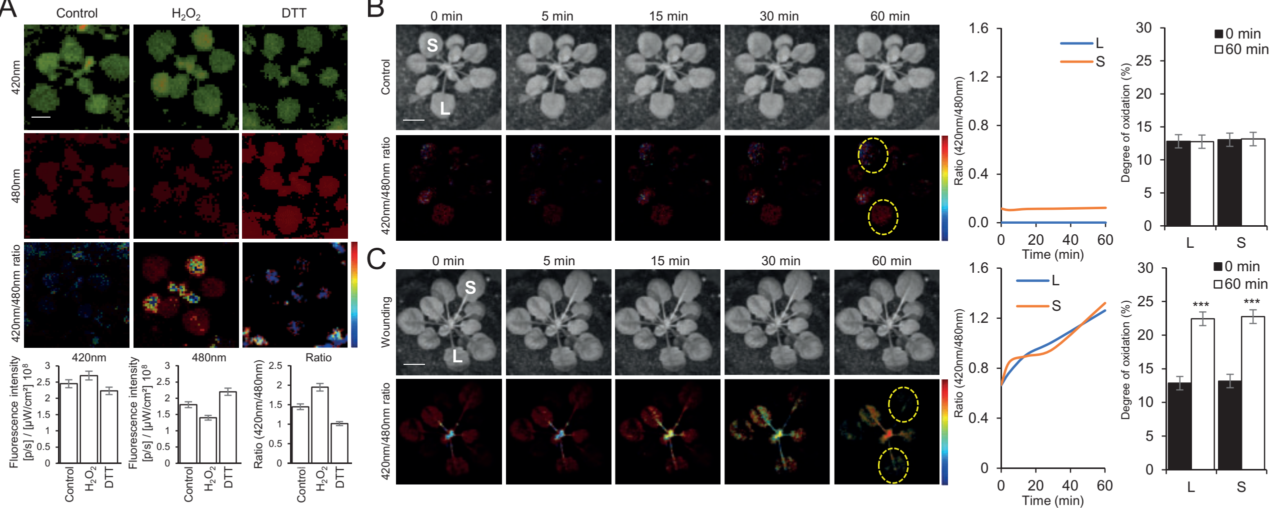

Figure 2 Whole-plant ratiometric fluorescence measurements of cytosolic roGFP1-expressing plants subjected to wounding. A, Representative images of whole-plant roGFP1 florescence (top; 420 and 480 nm excitations are in green and red, respectively), and statistical analysis of 420, 480, and 420/480 nm ratio (bottom) of untreated cytosolic roGFP1 plants (Control), or cytosolic roGFP1 plants subjected to a 15 min fumigation with 5 mM hydrogen peroxide (H_2O_2), or 5 mM DTT. Ratios of florescence intensities obtained from the H_2O_2 and DTT treatments were used for calculating the degree of roGFP1 oxidation (similar to Schwarzländer et al., 2008) shown in B. B, Representative time-lapse images (left), line graphs of 420/480 nm ratio for the entire time course (ROIs used to generate the graphs are shown in the images as dotted yellow ovals; middle), and bar graphs showing the degree of roGFP1 oxidation for the 0- and 60-min time points in local (L) and systemic (S) leaves calculated using the normalization range obtained in A, generated for the control untreated plants shown in Figure 1, B, top panels. C, Same as in B, but for the wounded plants shown in Figure 1, B, bottom panels. Experiments were conducted in three biological repeats, each with two technical repeats. Student's *t* test, SE, $N = 3$, $*P < 0.05$. Scale bar indicates 1 cm. L, local; S, systemic; ROI, region of interest.

glutathione redox levels because of its highly reduced state at the cytosol, which provides a low background in unstressed plants, and wounding as a local stimulus because treatments such as heat or high light stresses may alter roGFP1 activity. Wounding of a single leaf of an Arabidopsis (*Arabidopsis thaliana*) wild-type (Col-0) plant, grown in soil, resulted in the triggering of a systemic ROS wave response, imaged by the accumulation of oxidized 2′,7′-dichlorofluorescein (DCF) fumigated as H_2DCFDA into whole plants (Figure 1, A; Fichman et al., 2019). Wounding of a single leaf of a cytosolic-expressing roGFP1 transgenic plant (Jiang et al., 2006) grown in soil resulted in local and systemic changes in the redox state of the roGFP1 probe, evident by raw changes in excitation/emission at 420/520 nm (for oxidized roGFP1) and excitation/emission of 480/520 nm (for reduced roGFP1) fluorescence (Figure 1, B and Supplementary Movie S1). To quantify these changes and to calculate degree of oxidized roGFP1 in local and systemic tissues in response to wounding, we next determined the levels of 420/520 nm and 480/520 nm fluorescence in whole plants fumigated with 5 mM hydrogen peroxide (H_2O_2; to induce roGFP1 oxidation), or 5 mM dithiothreitol (DTT; to induce roGFP1 reduction), for 15 min and calculated the ratio of 420/480 nm for these treatments (Figure 2, A), and used these ratios to determine the 420/480 nm ratio and degree of roGFP1 oxidation in local and systemic leaves of the control and wounded plants shown in Figure 1 (Figure 2, B and C, and Supplementary Movies S2, S3). Wounding of a single Arabidopsis leaf resulted in the oxidation of the cytosolic roGFP1 probe in both local and systemic leaves (Figures 1, B, 2, C and Supplementary Movies S1–S3), and these changes corresponded to the changes recorded with the H_2DCFDA probe (Figure 1, A). Similar degrees of roGFP oxidation were recently reported for whole-plant imaging of chloroplastic roGFP2-expressing plants subjected to light stress (Haber and Rosenwasser, 2020), further supporting our findings and demonstrating that the degree of increase in roGFP oxidation in whole plants upon enhanced ROS accumulation is in the range of 5%–10% (Figure 2, C).

Our findings that wounding of a single leaf is accompanied by a systemic wave of ROS production (Fichman et al., 2019; Figure 1, A), as well as a systemic change in cytosolic redox levels (a "redox wave"; Figures 1, B, 2 and Supplementary Movies S1–S3) could therefore provide an initial clue to how the systemic ROS wave response alters the levels of metabolites and transcripts in systemic tissues causing an enhanced state of SAA or SWR (Miller et al., 2009; Szechyńska-Hebda et al., 2010; Suzuki et al., 2013; Gilroy et al., 2016; Fichman et al., 2019; Zandalinas et al., 2019, 2020a, 2020b). Interestingly, the roGFP1 probe remained oxidized for at least 60 min following wounding (Figure 1, B). This finding is in agreement with the systemic ROS wave response to excess light stress remaining "on" for at least 3 h following the initial application of a 10-min high light stress treatment to the local leaf (Devireddy et al., 2020). Further studies are of course needed to identify the different regulatory proteins altered by the ROS and redox

waves in response to a local stimulus and to tie their altered activity to the systemic response they cause. One potential regulator, recently identified during systemic responses to light stress, is MYB30 (Fichman et al., 2020). Because changes in MYB30 protein oxidation alter its DNA binding activity (Tavares et al., 2014), MYB30 could be a potential regulator that links changes in redox levels in systemic tissues to transcript expression and SAA (Fichman et al., 2020). In addition to demonstrating that the ROS wave is accompanied by a change in redox levels in local and systemic leaves, our study also demonstrates that at least some genetically encoded reporters can be imaged in whole-plants grown in soil, using a sufficiently sensitive apparatus (Fichman et al., 2019). This finding opens the way for further studies of ROS and redox signaling in whole plants grown in soil, and perhaps even to large-scale phenotyping studies.

Supplemental data

Supplementary Movie S1. Time-lapse video imaging of raw whole-plant changes in cytosolic roGFP1 fluorescence in response to wounding applied to the local leaf.

Supplementary Movie S2. Time-lapse video imaging of local and systemic changes in ratio of oxidized to reduced cytosolic roGFP1 in a control plant.

Supplementary Movie S3. Time-lapse video imaging of local and systemic changes in ratio of oxidized to reduced cytosolic roGFP1 in a wounded plant.

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

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