Singlet Oxygen-Induced Cell Death in Arabidopsis under High-Light Stress Is Controlled by OXI1 Kinase[1]

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Studies of the singlet oxygen ($^1O_2$)-overproducing *flu* and *chlorina1* (*ch1*) mutants of Arabidopsis (*Arabidopsis thaliana*) have shown that $^1O_2$-induced changes in gene expression can lead to either programmed cell death (PCD) or acclimation. A transcriptomic analysis of the *ch1* mutant has allowed the identification of genes whose expression is specifically affected by each phenomenon. One such gene is *OXIDATIVE SIGNAL INDUCIBLE1* (*OXI1*) encoding an AGC kinase that was noticeably induced by excess light energy and $^1O_2$ stress conditions leading to cell death. Photo-induced oxidative damage and cell death were drastically reduced in the OXI1 null mutant (*oxi1*) and in the double mutant *ch1*oxi1* compared with the wild type and the *ch1* single mutant, respectively. This occurred without any changes in the production rate of $^1O_2$ but was cancelled by exogenous applications of the phytohormone jasmonate. OXI1-mediated $^1O_2$ signaling appeared to operate through a different pathway from the previously characterized OXI1-dependent response to pathogens and H_2O_2 and was found to be independent of the EXECUTER proteins. In high-light-stressed plants, the *oxi1* mutation was associated with reduced jasmonate levels and with the up-regulation of genes encoding negative regulators of jasmonate signaling and PCD. Our results show that OXI1 is a new regulator of $^1O_2$-induced PCD, likely acting upstream of jasmonate.

Reactive oxygen species (ROS) are unavoidable by-products of photosynthesis in plants. Indeed, the photosynthetic processes involve electron-transfer mechanisms as well as generation of excited molecules, which can interact with molecular oxygen leading to the formation of reduced forms of oxygen such as superoxide and of singlet-excited oxygen ($^1O_2$; Apel and Hirt, 2004; Asada, 2006; Li et al., 2009). Superoxide can spontaneously or enzymatically disproportionate to hydrogen peroxide that can ultimately lead to the production of the hydroxyl radical in the presence of metals. These phenomena can be strongly amplified in plants exposed to environmental stress conditions that inhibit the photosynthetic activity and can lead to a situation where light energy is absorbed in excess to what can be used by the photosynthetic processes. The resulting over-reduction of the electron transport chain and increased lifetime of singlet-excited chlorophylls favor leakage of electrons and/or excitation energy to O_2, leading to enhanced ROS production. As a result, oxidative damage to the photosynthetic apparatus can occur, lowering photosynthetic efficiency and ultimately leading to cell death.

$^1O_2$ is produced within the photosystems (PS) from chlorophyll molecules in the triplet excited state (Krieger-Liszkay, 2005; Triantaphylidès and Havaux, 2009). $^1O_2$ is believed to be the main ROS produced in the chloroplasts under stress and excess light (González-Pérez et al., 2011) and to play a major destructive role during the execution of ROS-induced cell death in leaf tissues (Triantaphylidès et al., 2008). $^1O_2$ is a very reactive species that can readily oxidize macromolecules, particularly those containing double bonds (Triantaphylidès and Havaux, 2009). Because of its high reactivity, $^1O_2$ has a short lifetime in biological tissues, likely < 0.5 to 1 μs in plant cells (Bisby et al., 1999; Redmond and Kochevar, 2006; Li et al., 2012), making difficult the study of this ROS in planta. A major breakthrough in the study of $^1O_2$ effects on plants was the isolation and identification of

[1] This work was supported by the French National Research Agency through the SLOSAM project (ANR-14-CE02-0010). L.S. was supported by a CEA IRTELIS studentship.

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L.S., F.M., and M.H. designed the experiments; L.S. performed most experiments; A.C. performed some qRT-PCR analyses of gene expressions; L.T. performed the microarray-based transcriptomic analyses; B.L. performed the UPLC-MS analyses of jasmonates; L.S., F.M., and M.H. interpreted the data; L.S. and M.H. wrote the article.

www.plantphysiol.org/cgi/doi/10.1104/pp.15.01546

Arabidopsis (*Arabidopsis thaliana*) mutants that specifically produce $^1O_2$ under particular conditions. One such mutant is *flu*, a conditional mutant that accumulates a chlorophyll precursor in the dark (Meskauskiene et al., 2001). Upon transfer of *flu* seedlings from darkness to light, the accumulated chlorophyll precursor molecules act as photosensitizers in the light and bring about the production of high amounts of $^1O_2$ (op den Camp et al., 2003). The *flu* mutant appeared to be a very good experimental tool to generate $^1O_2$ in leaf tissues in a specific and relatively controlled manner. Thanks to this mutant, it was demonstrated that $^1O_2$ is not only a toxic molecule, but it can also function as a signal molecule inducing changes in gene expression that can lead to programmed cell death (PCD; op den Camp et al., 2003). This $^1O_2$ signaling pathway was found to be dependent on two chloroplastic proteins, EXECUTER1 (EX1) and EX2 (Wagner et al., 2004; Lee et al., 2007). Accordingly, $^1O_2$-induced cell death was suppressed in the *flu*ex1* double mutant deficient in the EX1 protein, and this took place without affecting the rate of $^1O_2$ formation.

Another $^1O_2$-overproducing Arabidopsis mutant, *chlorina1* (*ch1*), has emerged more recently as a useful tool to study noninvasively the in vivo signaling role of $^1O_2$ (Ramel et al., 2013a). Contrary to the *flu* mutant, this mutant produces $^1O_2$ in the PSII reaction centers, i.e. in the natural site of $^1O_2$ production in plants (Krieger-Liszkay, 2005; Ramel et al., 2012a). Because of the increased release of $^1O_2$ from the PSII centers, the *ch1* mutant is very photosensitive, exhibiting extensive leaf damage under light conditions that have little effect on the wild type (Havaux et al., 2007; Dall'Osto et al., 2010; Ramel et al., 2013a). The *ch1* mutant confirmed that $^1O_2$ can trigger changes in the expression of nuclear genes (Ramel et al., 2013a). Depending on the levels of $^1O_2$ production induced by light, the $^1O_2$-triggered signaling pathway in *ch1* was found to lead either to cell death or to an acclimation process (Ramel et al., 2013a). Transcriptomic analyses of *ch1* plants exposed to both conditions revealed marked difference in the gene expression profiles. Strikingly, most genes involved in the jasmonate biosynthesis pathway were strongly induced in *ch1* leaves under photooxidative stress conditions, while acclimatory conditions repressed those genes (Ramel et al., 2013a). Those contrasted changes in gene expression correlated with changes in the jasmonate concentrations in the leaves. Jasmonate, and possibly other phytohormones, appeared to be major players in the orientation of the $^1O_2$ signaling pathway toward a particular response (cell death or acclimation; Danon et al., 2005; Ramel et al., 2013b; Laloi and Havaux, 2015). A double mutant, *ch1*dde2*, that cannot synthesize jasmonate was found to be constitutively tolerant to photooxidative stress (Ramel et al., 2013b), affirming the function of jasmonate as a cell-death-promoting regulator under $^1O_2$ stress conditions (Laloi and Havaux, 2015).

Other remarkable differences in the transcriptome of *ch1* plants exposed to conditions leading to cell death or to acclimation concern genes known to play a role in the response to ROS and in cell death regulation. For instance, the gene encoding OXIDATIVE SIGNAL INDUCIBLE1 (OXI1) was observed to be specifically induced by stress conditions that led to cell death in *ch1* leaves (Ramel et al., 2013a). OXI1 is a Ser/Thr kinase that was shown to be regulated by H_2O_2 (Rentel et al., 2004). The *OXI1* gene is expressed in all plant organs and was principally localized at the cell periphery and in the nucleus (Anthony et al., 2004). This kinase was previously found to be necessary for ROS-mediated processes such as root hair growth (Rentel et al., 2004; Anthony et al., 2004) and plant-pathogen interactions (Rentel et al., 2004; Petersen et al., 2009; Camehl et al., 2011). OXI1 is believed to link oxidative burst signals and downstream responses through activation of mitogen-activated protein kinases (MAPKs; Rentel et al., 2004). This work was undertaken to investigate the possible implication of OXI1 in the responses of Arabidopsis to high-light stress and $^1O_2$. The results of this study reveal a crucial role for OXI1 in the control of $^1O_2$-induced PCD in Arabidopsis plants exposed to excess light energy, which operates by a mechanism different from the OXI1-dependent pathway triggered by H_2O_2 and pathogens.

RESULTS

Transcript Profile of the *OXI1* Gene in Arabidopsis Leaves under High-Light Stress

A previous microarray-based transcriptomic analysis of the $^1O_2$-overproducing Arabidopsis *ch1* mutant exposed to high-light stress (Ramel et al., 2013a) identified *OXI1* as a potentially interesting gene: it was markedly induced during light stress conditions that led to leaf damage but not differentially expressed during acclimation to $^1O_2$, suggesting that it might be a specific component of the $^1O_2$ signaling pathway leading to cell death. We have confirmed the microarray data for this gene using quantitative real-time PCR (qRT-PCR;

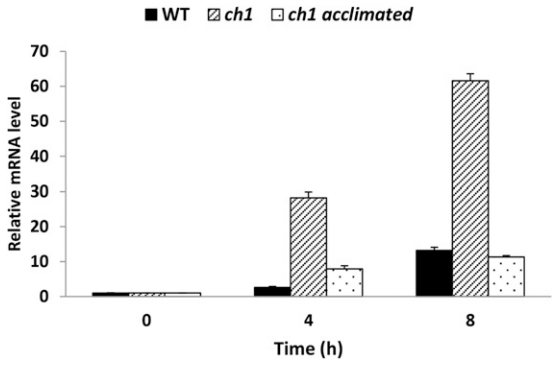

Figure 1. Transcript profile of the *OXI1* gene analyzed by qRT-PCR in wild-type Arabidopsis (WT) and in the *ch1* mutant (control or photoacclimated) exposed for 4 or 8 h to high-light stress (1500 μmol m^{-2} s^{-1} at 7°C/14°C, day/night). Photoacclimation was induced in the *ch1* mutant by a 2-d treatment at a moderately elevated PFD of 450 μmol m^{-2} s^{-1} at 20°C/18°C (day/night), as by Ramel et al. (2013a). Data are normalized to the values at time 0. Results are mean values of three biological replicates + SD.

Fig. 1). *OXI1* was induced in wild-type leaves during high-light stress: the transcript level was increased by a factor of 2.6 after 4 h in high light and a factor of 13 after 8 h. This induction was strongly enhanced in the *ch1* mutant (×30 and ×60 after 4 and 8 h, respectively) compared with the wild type, indicating high responsiveness of this gene to $^1O_2$ stress. The *ch1* mutant was also acclimated to high-light stress by pre-exposing the plants for 2 d to a moderately elevated photon flux density (PFD; 450 μmol m^{-2} s^{-1} at 20°C), as previously described (Ramel et al., 2013a). The acclimated *ch1* plants were then exposed to high-light stress for 4 or 8 h. Interestingly, photoacclimation of the *ch1* mutant blocked the induction of the *OXI1* gene after transfer to high-light stress (compared with *ch1* plants exposed to stress without acclimation) to similar values as in the wild type. Photoacclimation of *ch1* was previously shown not to modify the $^1O_2$ levels in leaves exposed to high PFDs (Ramel et al., 2013a). So, the low transcript levels in photoacclimated leaves are due to a down-regulation of the *OXI1* gene. The data in Figure 1 suggest a specific role of *OXI1* in photooxidative stress, with tolerance to $^1O_2$ stress being associated with inhibition of the up-regulation of this gene.

Photosensitivity of the Arabidopsis *oxi1* Mutant

An Arabidopsis mutant (*oxi1*) deficient in OXI1 was previously studied in the context of pathogen attacks (Camehl et al., 2011). Although OXI1 is required for normal root development (Rentel et al., 2004; Anthony et al., 2004), *oxi1* mutant shoots did not exhibit any growth phenotype under normal growth conditions compared with its wild type (not shown, but see Figure 2B, panel i, for rosette size). We crossed this mutant with the *ch1* mutant to generate a $^1O_2$-overproducing, OXI1-deficient double mutant (*ch1*oxi1*). Compared with *ch1*, the *ch1*oxi1* double mutant did not show any alteration of its shoot-growth capacities under normal conditions. *Ch1* and *ch1*oxi1* mutants were transferred from normal growth light (PFD 120 μmol photons m^{-2} s^{-1}) to high-light stress (1100 μmol m^{-2} s^{-1}). Air temperature was simultaneously decreased to 7°C to prevent overheating of the leaves during high-light stress and to maintain leaf temperature in the range of the control leaf temperature (~18°C). As shown in Figure 2A (panel i, top), *ch1*oxi1* plants appeared to be more resistant to high-light stress than *ch1* plants as depicted by decreased leaf bleaching. In leaves, lipids are highly unsaturated (Douce and Joyard, 1980) and therefore very sensitive to oxidation (Mène-Saffrané et al., 2009; Farmer and Mueller, 2013). Consequently, photooxidative damage to plant leaves can be estimated by measuring the extent of lipid peroxidation. Accumulation of lipid peroxides can be visualized via the light emission associated with their slow spontaneous decomposition (Havaux et al., 2006; Birtic et al., 2011). Figure 2A (panel i, bottom) shows that the autoluminescence of high-light-treated *ch1*oxi1* plants was substantially lower than the luminescence signal emitted by *ch1* plants, indicating a lower

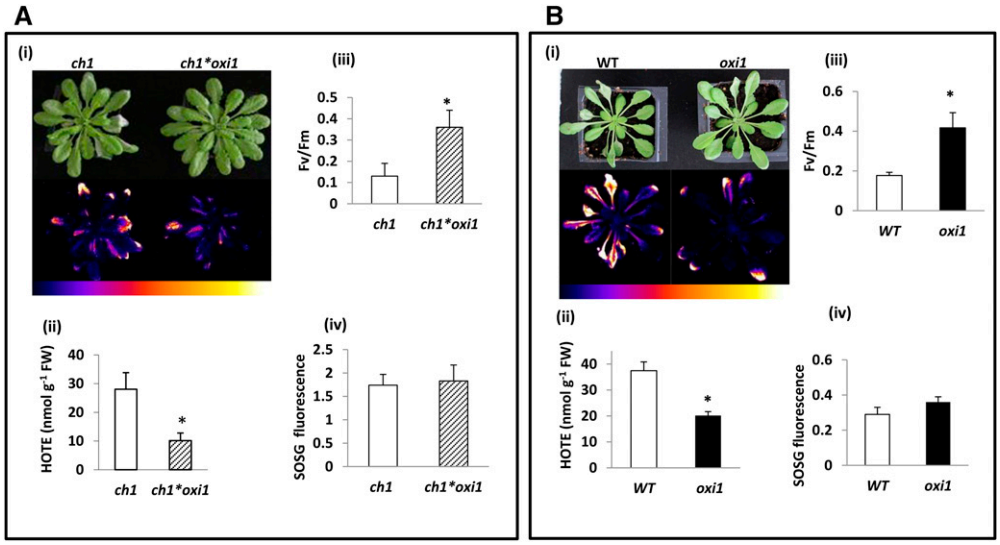

Figure 2. Effect of the *oxi1* mutation on the tolerance to high-light stress of $^1O_2$-overproducing *ch1* mutant plants (A) and wild-type Arabidopsis plants (WT; B). The high-light treatments were 2 d at 1100 and 1500 μmol m^{-2} s^{-1} at 7°C/14°C (day/night) for A and B, respectively. i, Picture of the plants after the high-light stress treatment (top) and autoluminescence imaging of lipid peroxidation (bottom). Color scale indicates signal intensity from 0 (blue) to saturation (white). ii, Lipid peroxidation measured by HPLC quantification of hydroxy fatty acids (HOTE) after high-light stress. iii, PSII photochemical efficiency as measured by the F_v/F_m chlorophyll fluorescence ratio after high-light stress. iv, $^1O_2$ production measured in leaves by fluorescence of the SOSG probe after exposure of SOSG-infiltrated plants to high light for 30 min. Background fluorescence produced when SOSG-infiltrated plants were placed in the dark for 30 min was subtracted from fluorescence produced in light. Asterisk indicates significant difference between *oxi1*/*ch1*oxi1* plants and wild-type/*ch1* plants, respectively, at $P < 0.05$ (Student's *t* test). Results are mean values of five measurements (ii) and 10 measurements (iii and iv) + SD.

accumulation of lipid peroxides in the former plants after high-light stress. Differential lipid peroxidation was confirmed by HPLC analyses of hydroxyoctatrienoic acid (HOTE; the oxidation product of linolenic acid C18:3, the major fatty acid in Arabidopsis leaves), which showed drastic decrease in the *ch1*oxi1* double mutant relative to the *ch1* single mutant (Fig. 2A, panel ii). The decrease in lipid peroxidation in *ch1*oxi1* relative to *ch1* correlated with a lesser diminution of the PSII photochemical efficiency measured by the F_v/F_m chlorophyll fluorescence ratio (Fig. 2A, panel iii).

We also compared the responses of the wild type and the *oxi1* single mutant to high-light stress (Fig. 2B). Because wild-type plants are much more tolerant to high-light stress compared with *ch1* plants (Ramel et al., 2013a), it is necessary to use a higher PFD (1500 μmol m^{-2} s^{-1}) to achieve comparable stress intensities. Similar to what we observed in the *ch1* genetic background, the *oxi1* mutation showed decreased leaf photodamage, with reduction of leaf bleaching (panel i), decrease in lipid peroxidation as measured by autoluminescence imaging (panel i) and HOTE levels (panel ii), and more preserved PSII activity (panel iii) after high-light stress.

To ascertain the fact that the observed photoresistant phenotype of *oxi1* and *ch1*oxi1* mutants under high-light stress compared with the wild type and *ch1*, respectively, was not imputable to different levels of $^1O_2$ production, $^1O_2$ produced in leaves after 30 min of high-light treatment was measured by fluorescence of the $^1O_2$-specific Singlet Oxygen Sensor Green (SOSG) fluorescent probe (Flors et al., 2006). No difference in $^1O_2$ production was observed between *ch1* and *ch1*oxi1* (Fig. 2A, panel iv) or between the wild type and *oxi1* (Fig. 2B, panel iv). However, as expected (Dall'Osto et al., 2010; Ramel et al., 2013a), $^1O_2$ accumulation in the *ch1* background (Fig. 2A, panel iv) was noticeably higher than the production in the wild-type background (Fig. 2B, panel iv). A drawback of SOSG is that it can act as a photosensitizer producing $^1O_2$ upon exposure to UV radiation and, to a lesser extent, visible light (Ragàs et al., 2009). We exposed a SOSG solution to the light conditions used in this study (1500 μmol photons m^{-2} s^{-1} for 30 min), and we observed that SOSG fluorescence at 525 nm was slightly increased (Supplemental Fig. S1), confirming the idea that some $^1O_2$ photosensitization by SOSG can occur upon illumination. However, this light-induced increase in SOSG fluorescence was low compared with the effect of $^1O_2$ produced by Rose Bengal. Moreover, this phenomenon is likely to be minor in our analyses of $^1O_2$ production by Arabidopsis leaves. Indeed, SOSG fluorescence was much higher in *ch1* leaves compared with wild-type leaves (Fig. 2, A, panel iv, and B, panel iv), although the light treatment was less intense, indicating that this difference cannot be merely due to a direct effect of light on SOSG. In addition, the comparison between the wild type and *oxi1* or between *ch1* and *ch1*oxi1* in Figure 2 was done at the same PFD (1500 or 1100 μmol m^{-2} s^{-1}, respectively), and, therefore, the interference of $^1O_2$ photosensitization by SOSG, if any, can be supposed to be the same for both genotypes in each comparison. Nevertheless, the SOSG data must be considered as semiquantitative only. Based on the results presented in Figure 2, OXI1 thus appeared to play a crucial role in the development of cellular damage during high-light stress in Arabidopsis.

OXI1-Mediated Cell Death in Arabidopsis Is Independent of the EX Proteins

PCD is used by plants as a means of defense against many ROS-mediated stresses (Van Breusegem and Dat, 2006; Gadjev et al., 2008). One peculiar feature of ROS-mediated PCD is that the ROS triggers reprogramming of expression of defense genes. In *flu*, EX1 and EX2 are principal mediators of $^1O_2$-triggered cell death (Wagner et al., 2004; Lee et al., 2007). Cell death and up-regulation of almost all $^1O_2$-specific genes induced by a dark-to-light transfer in the *flu* mutant were abolished in the *flu*ex1*ex2* triple mutant (Lee et al., 2007). However, our study showed that the EX proteins do not mediate PCD in the wild-type Columbia (Col)-0 genetic background under conditions of excess light energy induced by high PFD and low temperature: *ex1*ex2* double mutant plants showed similar susceptibility to high-light stress as wild-type plants, depicted by similar levels of leaf bleaching and lipid peroxidation measured by autoluminescence imaging (Fig. 3A) and HOTE levels (Fig. 3B).

To analyze the implication of OXI1 and EX1 and EX2 in the process of $^1O_2$-mediated cell death, we measured cell death in mature leaves of *oxi1* and *ex1*ex2* mutants by Evans blue staining relative to the wild type (Fig. 4). As shown in Figure 4A, wild-type leaves retained more dye than *oxi1* mutant leaves, indicating an increased extent of cell death in the former leaves. This phenomenon was quantified in Figure 4B, revealing an almost doubling of the amount of dye retained in wild-type leaves relative to *oxi1* leaves. This confirms the role of OXI1 in high-light-induced PCD in Arabidopsis. On the contrary, *ex1*ex2* double mutant leaves retained similar amounts of the dye as wild-type leaves, thus strengthening the irrelevance of the EX proteins in cell death mediation in the Arabidopsis Col-0 background during high-light stress.

The $^1O_2$ Signaling Pathway Is Different from the OXI1-Dependent H_2O_2 Pathway Involving MAPKs

OXI1 is known to be essential for the full activation of MAPK3 and MAPK6 in plants submitted to pathogen attacks (Rentel et al., 2004). Activation of *OXI1* and downstream components of the signaling pathway are known to be achieved by sequential phosphorylation. The presumed pathway for OXI1 signaling during pathogen attack has previously been established (Hirt et al., 2011), as illustrated in Supplemental Figure S2. This pathway indicates PDK1-dependent

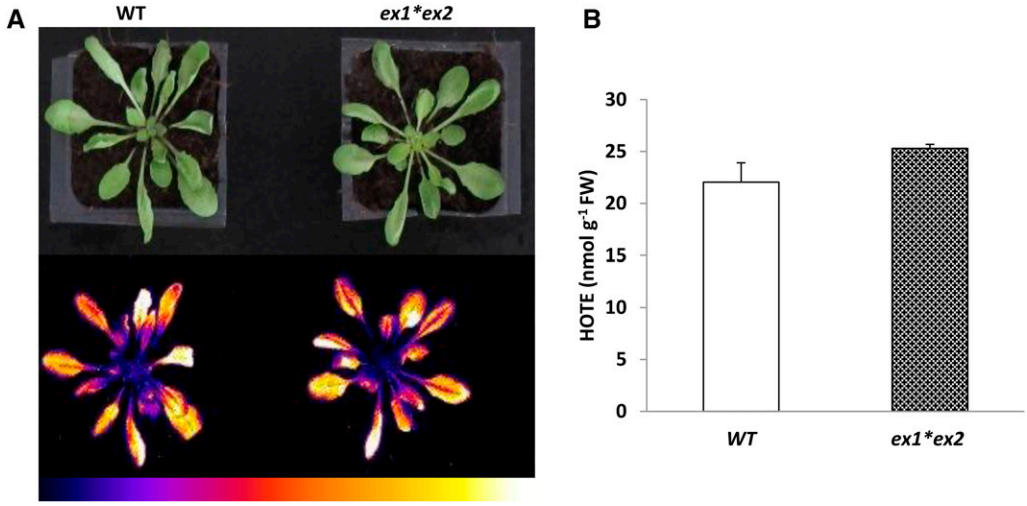

Figure 3. Lipid peroxidation in wild-type Arabidopsis (WT) and in the *ex1*ex2* double mutant after high-light stress (2 d at 1500 μmol m^{-2} s^{-1} and 7°C/14°C, day/night). A, Autoluminescence imaging of lipid peroxides. B, HPLC quantification of hydroxy fatty acid (HOTE). Data are mean values of five measurements + SD.

and PDK1-independent routes for activation of *OXI1* by pathogens. The major ROS triggering the signaling during pathogen attack is H_2O_2. We were interested to determine if the OXI1 pathway for PCD triggered by $^1O_2$ is similar to that portrayed during H_2O_2 stress. Whole wild-type and *oxi1* plants were sprayed with 5% H_2O_2 and allowed for 2 h under normal growth conditions (140 μmol photons m^{-2} s^{-1} light and 20°C/18°C day/night). On the other hand, $^1O_2$-specific stress was induced by exposing *ch1* and *ch1*oxi1* plants to high-light stress (1100 μmol photons m^{-2} s^{-1}). The general transcript profiles of the OXI1-dependent signaling pathway showed great differences between H_2O_2 treatment and $^1O_2$ stimulation as illustrated in Figure 5. Both H_2O_2 and $^1O_2$ induced *OXI1* expression and repressed the upstream activator of *OXI1*, *PDK1* (At5g04510). As expected (Rentel et al., 2004), activation of *OXI1* by H_2O_2 led to an increase in transcript levels for the downstream components *PTI1-4* (At2g47060), *PTI1-1* (At1g06700), *MAPK3* (At3g45640), and *MAPK6* (At2g43790) in the wild type. In the *oxi1* mutant, the H_2O_2-induced changes in these downstream components were perturbed as previously shown (Rentel et al., 2004): *PTI1-4*, *PTI1-1*, and *MAPK6* were slightly repressed (instead of being induced), while *MAPK3* was not fully activated. In striking contrast with the H_2O_2-induced changes in gene expressions, neither *PTI-1* and *PTI1-4* nor *MAPK3* and *MAPK6* were induced by $^1O_2$ in the *ch1* mutant exposed to high light, and the *oxi1* mutation in the *ch1*oxi1* double mutant did not affect these responses (Fig. 5). The differential response of the *PTI1* and *MAPK* genes between H_2O_2 and $^1O_2$ treatments and the similar responses portrayed by these genes in the *ch1* and *ch1*oxi1* mutants clearly indicate that $^1O_2$-induced signaling for cell death regulated by OXI1 occurs by a pathway different from that of the OXI1-mediated signaling pathway induced by pathogen attack and H_2O_2 stress, which involves *MAPK3* and *MAPK6* activation.

Microarray-Based Transcriptomic Analysis of the *oxi1* Mutant under Photooxidative Stress Conditions

To gain more insight into the effect of *oxi1* mutation during high-light stress, a transcriptomic analysis was performed on *oxi1* mutant plants compared with wild-type plants, using the CATMAv6 microarray system (Hilson et al., 2004). Three comparisons were performed: *oxi1*-control versus wild type-control to identify transcripts affected as a result of mutation in the *OXI1* gene; *oxi1*-stress versus wild type-stress to dissect transcripts affected in the *oxi1* mutant under stress, which may explain the phenotype observed when compared with the wild type; and *oxi1*-stress versus *oxi1*-control to identify transcripts affected in the *oxi1* mutant specifically due to high-light stress. Among the 38,356 genes identified in the CATMAv6 array, 1221 genes were differentially expressed (with P value $<$ 0.05) in the *oxi1*-control versus wild type-control comparison, corresponding to 3.6% of the total genes. This comprised 1.5% genes up-regulated and 2.1% genes down-regulated (Fig. 6A). Functional classification of these differentially expressed genes using the DAVID gene functional classification tool revealed that the mutation affects a number of important biological and metabolic processes, such as response to endogenous stimuli, ethylene-mediated signaling pathway, and toxin metabolic processes that were repressed. Glucosinolate and anthocyanin biosynthetic processes, carbohydrate biosynthetic processes, responses to endogenous stimuli (ethylene, jasmonic acid, etc.), and lipid transferase activity were induced (Supplemental Table S1).

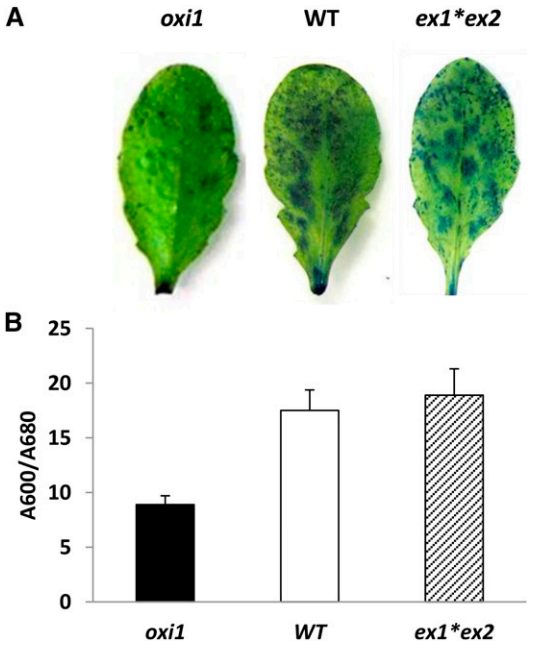

Figure 4. High-light-induced cell death measured in leaves by Evans blue staining. Wild-type Arabidopsis (WT) is compared with the single mutant *oxi1* and the double mutant *ex1*ex2*. The high-light treatment was 2 d at 1500 μmol photons m^{-2} s^{-1} and 7°C/14°C (day/night). A, Visualization of dead cells in leaves which retain the Evans blue stain. B, Quantification of cell death in leaves by measuring the absorbance ratio (A_{600}/A_{680}) of the Evans blue stain solution extracted from the leaves. Data are mean values of five measurements + SD.

Comparing *oxi1*-stress versus *oxi1*-control, 3262 genes were differentially expressed (with P value $<$ 0.05), corresponding to 4% up-regulated and 5.7% down-regulated genes (Fig. 6A). Functional classification of genes differentially expressed using the DAVID tool revealed that the down-regulated genes coded for proteins involved in processes such as response to endogenous/hormone stimuli, glucosinolate biosynthesis/metabolism, response to abiotic stress, photosynthesis, and oxylipin/jasmonate biosynthesis/metabolism (Supplemental Table S2). Interestingly, most of the genes of the jasmonate biosynthetic pathway, including *LOX3* (At1g17420), *LOX2* (At3g45140), *AOS* (At5g42650), *OPR3* (At2g06050), *AOC1* (At3g25760), *JMT* (At1g19640), and *AOC4* (At1g13280), were down-regulated. This finding shows a behavior very different from the responses to $^1O_2$ in the *flu* and *ch1* mutants, which have been shown to be associated with a strong induction of the jasmonate pathway (Przybyla et al., 2008; Ramel et al., 2013a). Considering the fact that jasmonate is associated with $^1O_2$-induced cell death in the *flu* and *ch1* mutants (Laloi and Havaux, 2015), this finding suggests that the OXI1 action during high-light stress could involve a regulation of jasmonate synthesis and/or activity. The genes up-regulated in *oxi1* by high-light stress were mainly involved in the processes of ribonucleoprotein and cellular protein biosynthesis, flavonoid biosynthesis, helicase activity, ATPase activity, toxin metabolism, and response to abiotic stimuli, represented by overexpression of genes implicated in detoxification processes, such as glutaredoxin-C14 (At3g62960), Peroxiredoxin-C2 (At1g65970), glutathione *S*-transferases (*ATGSTU1* [At2g29490], *ATGSTU11* [At1g69930], and *ATGSTU16* [At1g59700]), *CAT2* (At4g35090), *HSP70* (At3g12580), and the transcription factors *HY5* (At5g11260) and *MYB32* (At4g34990; Supplemental Table S2).

Of great importance to us was to assess the transcript profile of the *oxi1* mutant relative to the wild type under stress conditions, which was achieved by the comparison *oxi1*-stress versus wild type-stress in the microarray analysis. A total of 1568 genes were differentially expressed after stress (P value $<$ 0.05), which account for 4.7% of the total genes analyzed (Fig. 6A), with up-regulation and down-regulation representing half of the 1568 genes each (Fig. 6B). The majority of the genes up-regulated and down-regulated in this comparison were found to have $\log_2$ intensity ratios between 1 and 2, which corresponds to 2- to 4-fold up-regulation/down-regulation of the genes in the stressed *oxi1* mutant compared with stressed wild-type plants (Fig. 6C). As observed in the *oxi1*-stress versus *oxi1*-control comparison, functional classification of down-regulated genes using the DAVID functional classification tool also revealed down-regulation of genes implicated in the response to hormonal stimuli, mainly auxin, as represented by genes such as *PIN4* (At2g01420), *AUX1* (At2g38120), *IAA15* (At4g14560), and *IAA14* (At4g14550); ethylene, as depicted by genes such as *DREBA1A* (At4g25480), *DREBA2B* (At3g11020), *ERF8* (At1g53170), and *ERF015* (At4g31060); and gibberellic acid, as represented by genes such as *RGA1* (At2g01570), *GA2* (At1g79460), *GA3* (At5g25900), and *GASA4* (At5g15230; Supplemental Table S3). Other processes in this category included response to abiotic stress, photosynthesis, oxidoreductases, and organic acid biosynthetic processes (Supplemental Table S3). As far as the jasmonate biosynthesis pathway is concerned, repression in high-light-stressed *oxi1* leaves compared with stressed wild-type leaves concerned mainly the *AOC4* gene (At1g13280). Also, similar to up-regulation in the *oxi1*-stress versus *oxi1*-control comparison, we observed an up-regulation of genes coding for specific processes, such as ribosome biogenesis, response to abiotic stimuli (genes such as *CAT2* [At4g35090], *HSP70* [At3g12580], and *HY5* transcription factor [At5g11260] involved in ROS-dependent stress regulation), flavonoid, anthocyanin, and other pigment biosynthesis. In addition, there was up-regulation of genes related to salicylic acid, such as *GRP23* (At1g10270), *PBS3* (At5g13320), *MYB77* (At3g50060), and *CCA1* (At2g46830), as well as genes involved in response to jasmonate, predominantly the TIFY genes that code for the JAZ proteins (*JAZ5* [At1g17380], *JAZ6* [At1g72450], *JAZ7* [At2g34600], and *JAZ10* [At5g13220]), which are negative regulators of jasmonate (Supplemental Table S3). To sum up, relative to the wild type, high-light stress in *oxi1* leaves causes significant down-regulation of photosynthesis, growth processes, and phytohormones, and up-regulation of structural component biogenesis

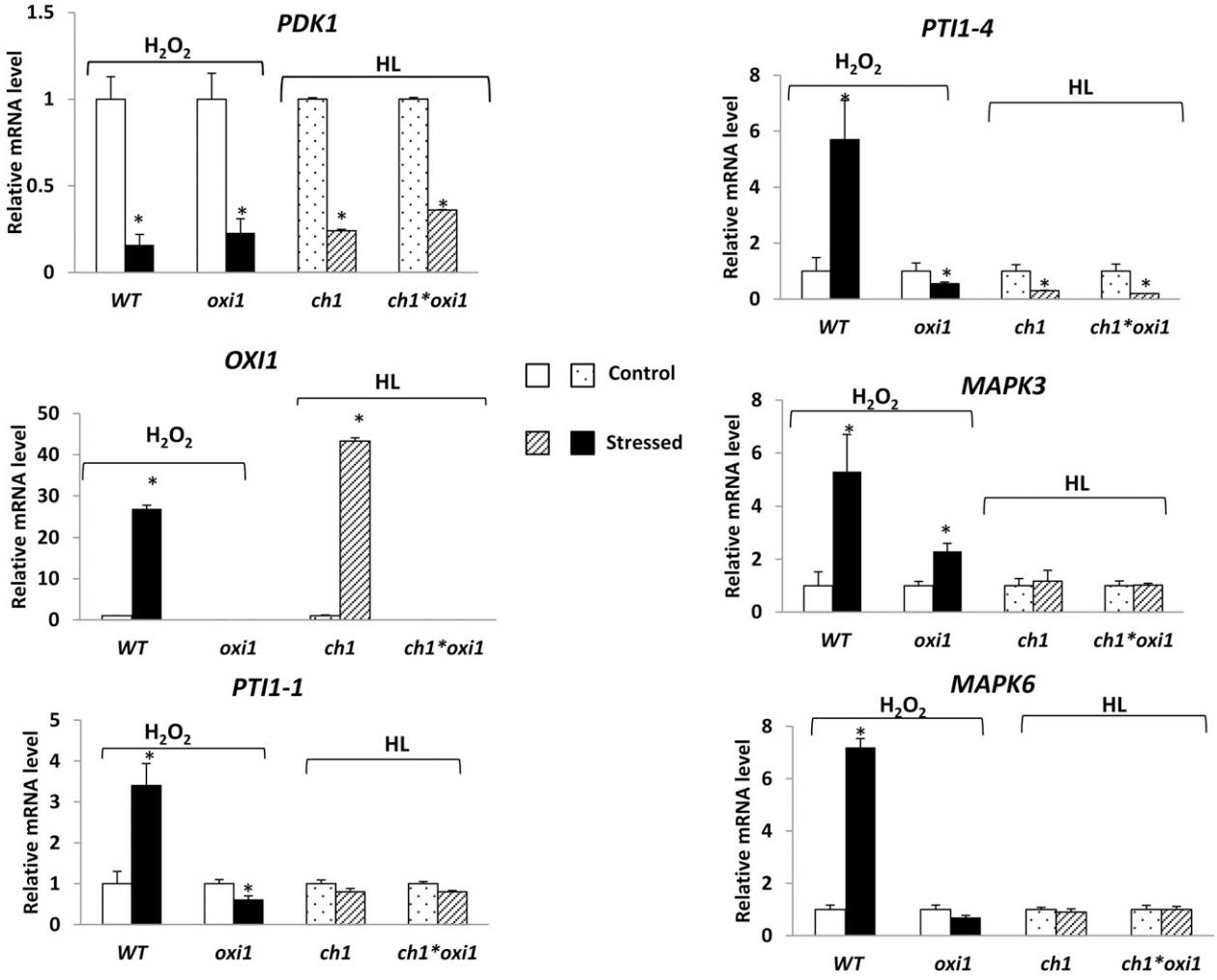

Figure 5. Transcript profiling of genes involved in the OXI1-dependent signaling pathway (*PDK1*, *OXI1*, *PTI1-1*, *PTI1-4*, *MAPK3*, and *MAPK6*), during H_2O_2 stress compared with $^1O_2$ stress. H_2O_2 stress was generated by spraying wild-type (WT) and *oxi1* mutant plants with 5% H_2O_2 and incubating for 2 h in low light (150 μmol photons m^{-2} s^{-1}). $^1O_2$ stress was generated by exposure of *ch1* and *ch1*oxi1* plants to high-light stress (HL, 1100 μmol photons m^{-2} s^{-1} at 7°C). Asterisk indicates significant difference in gene expression between wild type and *oxi1* and between *ch1* and *ch1*oxi1* at $P < 0.05$ (Student's *t* test). Data are mean values of three biological replicates + SD.

and pigment biosynthesis, all of which are regulated by an interplay of the hormones auxin, ethylene, jasmonic acid, salicylic acid, and gibberellins.

Interestingly, in the transcriptomic comparison of *oxi1* and the wild type, we noticed the up-regulation in *oxi1* leaves of a number of genes proposed to antagonize cell death processes (Cominelli et al., 2000; Danon et al., 2004; Alonso-Peral et al., 2010). This was confirmed by qRT-PCR analyses (Fig. 6D): *MYB90* (At1g66390), *DAD1* (At1g32210), and *MYB114* (At1g66380) were up-regulated in stressed *oxi1* plants compared with wild-type plants. These gene expression changes are consistent with the phenotype of the mutant that is more resistant to high light and less prone to cell death than the wild type (Figs. 1 and 4). This observation raises the possibility that *OXI1* is a master regulatory gene that controls PCD induced by $^1O_2$ stress.

Interactions between Jasmonate and OXI1-Dependent Signaling

The significant down-regulation of jasmonate-related genes in high-light-stressed *oxi1* mutant plants observed in the transcriptome studies suggests a possible involvement of decreased levels of jasmonic acid in cell death attenuation in this mutant. In line with this idea, *oxi1* plants after exposure to high-light stress for ~ 26 h portrayed ~ 2-fold lower levels of jasmonic acid and jasmonyl-Ile (JA-Ile) compared with wild-type plants (Fig. 7A). In contrast, the levels of the jasmonic acid precursor oxophytodienoic acid (OPDA) were similar in both wild-type and *oxi1* plants.

We also examined the effect of exogenous jasmonate applications on the response of the *oxi1* mutant to high-light stress (Fig. 7). *Oxi1* plants recovered the susceptibility

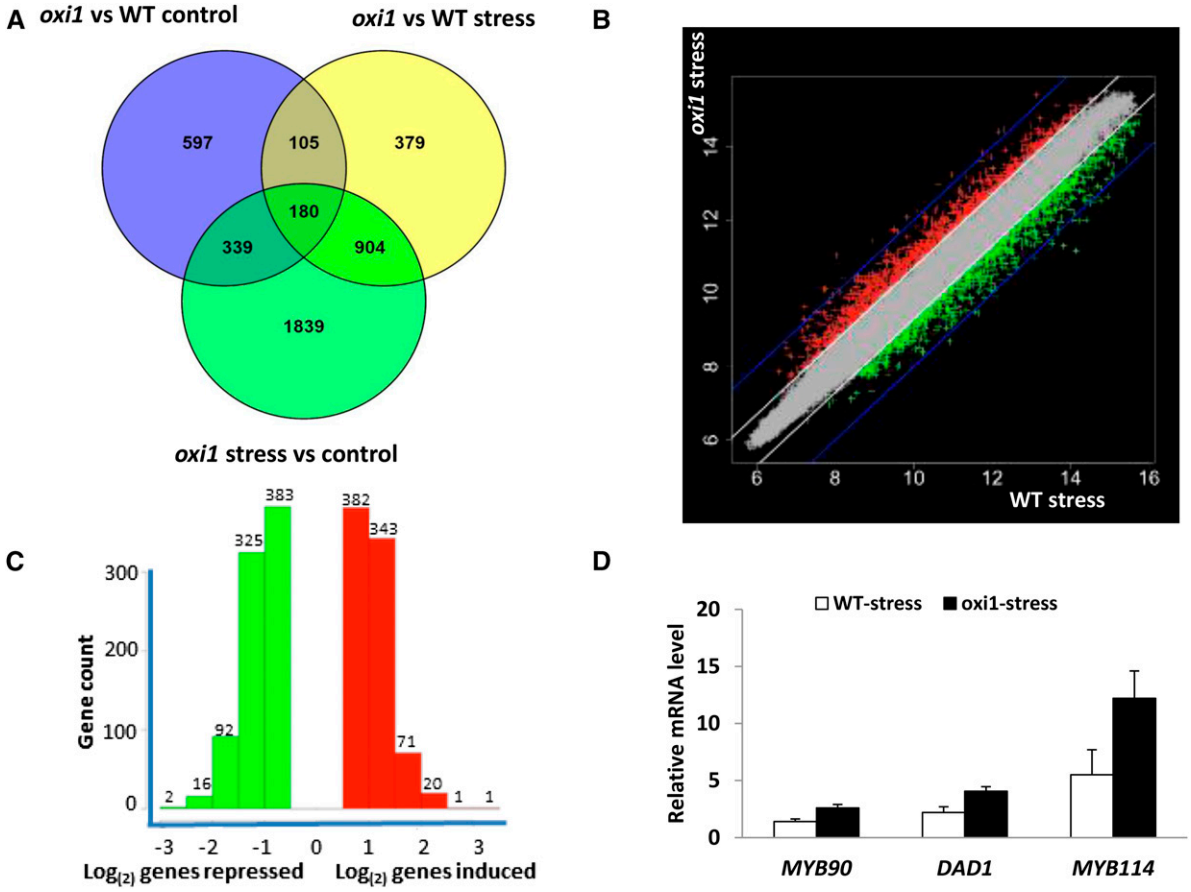

Figure 6. Microarray analysis of the transcriptome of *oxi1* relative to the wild type (WT). A, Venn diagram showing the number of genes differentially expressed in three comparisons, as well as genes commonly expressed in the different comparisons. B, Plot of mean $\log_2$ signal intensities of genes differentially induced (red), repressed (green), and nondifferentially expressed (gray) at $P < 0.05$, under high-light-stress conditions in *oxi1* mutant relative to the wild type. Signal intensities < 7 correspond to genes that are very weakly expressed, between 7 and 10 are weakly to averagely expressed, > 10 are highly expressed, and > 13 are the most abundant transcripts. Points above and below the blue lines represent genes in *oxi1* with respectively $>$ and < 2-fold intensities compared with the wild type after stress. C, Distribution of genes induced (red) and repressed (green) in the wild type-stress versus *oxi1*-stress comparison by the $\log_2$ ratio of folds expression. Negative sign represents repression. D, qRT-PCR profile of genes of apoptosis regulators (*MYB90* [At1g66390], *DAD1* [At1g32210], and *MYB114* [At1g66380]) identified in the transcriptome as differentially expressed in *oxi1* and the wild type. Data are mean values of three biological replicates + SD.

phenotype of wild-type plants under high-light stress when sprayed with methyl-jasmonate, as illustrated by the autoluminescence images of lipid peroxidation shown in Figure 7B. This also corresponded to a similar accumulation of HOTE in the *oxi1* mutant and the wild type (Fig. 7D). These findings indicate enhanced stress in the *oxi1* mutant after high-light exposure in the presence of exogenously applied methyl-jasmonate compared with the substantial reduction of lipid peroxidation in *oxi1* relative to the wild type observed when the high-light treatment was imposed without applying exogenous jasmonate (Fig. 2B, panel ii). The effects of exogenous jasmonate were also observed in the *ch1* genetic background: the phototolerance of the *ch1*oxi1* double mutant was abolished by the jasmonate treatment (compare Figs. 7C and 2A; see also Fig. 7D). Spraying wild-type, *oxi1*, *ch1*, or *ch1*oxi1* plants with methyl-jasmonate in low light did not induce leaf damage or lipid peroxidation, excluding a direct toxicity of jasmonate on the plants (data not shown).

DISCUSSION

OXI1 Is a New Regulator of High-Light-Induced Cell Death in Arabidopsis

Singlet oxygen is generated from triplet excited chlorophyll molecules and is therefore intimately associated with light absorption and photosynthesis in plants. $^1O_2$ has been shown to be the main ROS produced in plant cells in high light (González-Pérez et al., 2011) and the major ROS involved in photooxidative damage to plant leaves (Triantaphylidès et al., 2008). This study has identified OXI1 as a link between $^1O_2$ production in the chloroplasts under excess light

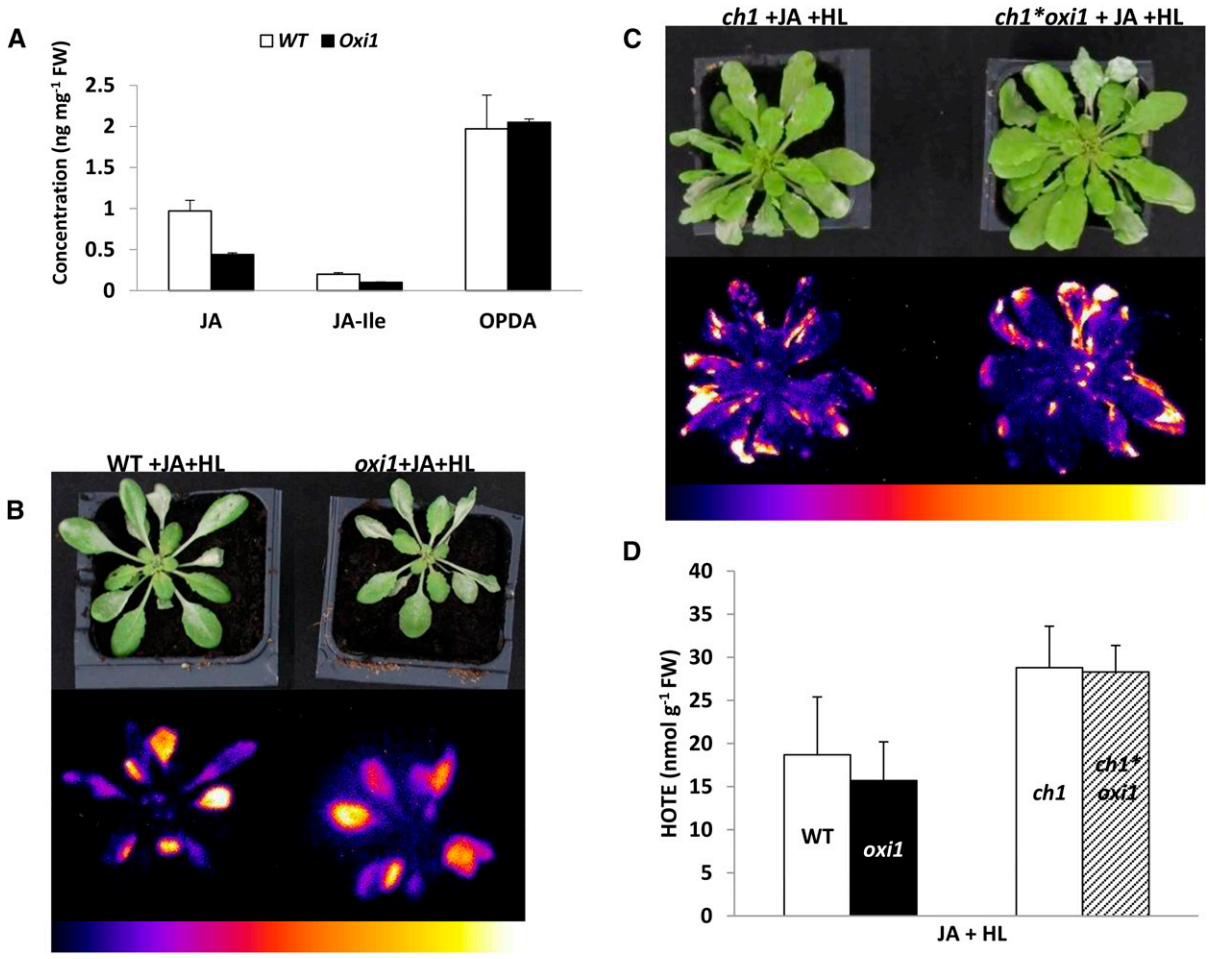

Figure 7. Implication of jasmonate in OXI1-mediated response to photooxidative stress. A, UPLC-MS quantification of jasmonic acid (JA), its active form JA-Ile, and its precursor OPDA, from wild-type (WT) and *oxi1* plants exposed to 26 h of high-light and low-temperature stress (1500 μmol m^{-2} s^{-1} and 7°C). Data are mean values of five measurements + SD. B, Autoluminescence image of lipid peroxidation of wild-type and *oxi1* plants sprayed with methyl-jasmonate under high-light and low-temperature stress (JA+HL). C, Autoluminescence imaging of *ch1* and *ch1*oxi1* mutant plants sprayed with methyl-jasmonate (JA + HL) under high-light and low-temperature stress conditions (1100 μmol m^{-2} s^{-1} and 7°C). Color scales indicate signal intensity from 0 (blue) to saturation (white). D, HPLC measurements of hydroxy fatty acids (HOTE). Data are mean values of at least four measurements + SD.

energy and the resulting development of leaf cell death. The *OXI1* gene was strongly induced by $^1O_2$ (Figs. 1 and 5), and mutational suppression of OXI1 in the *oxi1* null mutant drastically reduced the extent of photooxidative damage and cell death in high-light-stressed leaves (Fig. 2). This occurred without any change in the $^1O_2$ accumulation levels, indicating that OXI1 is required for the $^1O_2$ signaling process leading to cell death. Moreover, acclimatory conditions that increase tolerance to $^1O_2$ stress and prevent photooxidative damage blocked the $^1O_2$-induced up-regulation of *OXI1*. Thus, this study confirms the involvement of OXI1 kinase in ROS signaling and extends the area of intervention of this protein, which was previously limited to immune responses to pathogens, into high-light and $^1O_2$ stress. Incidentally, because the extent of photoinduced cell death was noticeably reduced by the *oxi1* mutation, this work also confirms in a mutant different from *flu* that the damaging effects of $^1O_2$ involve a genetic program and result only partially from the direct toxicity of this ROS.

Regulation of $^1O_2$-induced cell death by OXI1 resembles the function previously attributed to the EX proteins in the *flu* mutant (Wagner et al., 2004; Lee et al., 2007). PCD induced in this mutant by a dark-to-light transition was drastically reduced when EX1 and/or EX2 was inactivated. However, the signaling pathway that leads to cell death in wild-type plants under high-light stress was found here to be independent of the EX proteins (Fig. 3). Previously, the gene expression profile induced by $^1O_2$ in the *ch1* mutant was also observed to be unaffected by the *ex1* mutation (Ramel et al., 2013a). Similarly, a recent study of Arabidopsis cell suspensions treated with the $^1O_2$ generator Rose Bengal has shown that the induced changes in gene expression led to PCD by a mechanism that appears to be distinct from

the EX-dependent pathway described in *flu* (Gutiérrez et al., 2014). Thus, the role of the EX proteins as cell death modulators does not apply to all conditions that promote $^1O_2$ production.

A major difference between *ch1* (and the wild type) and *flu* is the site of $^1O_2$ production: in the *flu* mutant, $^1O_2$ is formed in thylakoid membranes from the chlorophyll precursor protochlorophyllide that does not accumulate normally in wild-type membranes while it is formed from chlorophyll in the photosynthetic complexes in wild-type and *ch1* leaves. One can then speculate that the signaling pathway of $^1O_2$ depends on how and where $^1O_2$ is formed in leaf cells. It is also important to mention that $^1O_2$ can oxidize the β-carotene molecules located in the PSII reaction centers, producing various oxidized derivatives (Ramel et al., 2012a). Some of those β-carotene metabolites were shown to act as upstream messengers in the gene responses leading to acclimation to $^1O_2$ (Ramel et al., 2012b; Shumbe et al., 2014). Similarly, $^1O_2$ oxidation of protochlorophyllide in *flu* mutant leaves could possibly generate oxidized molecules that are not normally present in the wild type, and we cannot exclude an effect of these metabolites in gene responses. Consistently, it is known that porphyrins can operate as cell death regulators, even in the unexcited state (Hirashima et al., 2009). Thus, different signal molecules may be generated in the *ch1* and *flu* mutants, possibly leading to different signaling pathways. In agreement with our results, Kim and Apel (2013) reported an EX-dependent $^1O_2$ signaling in wild-type Arabidopsis under mild conditions only; more severe conditions that lead to photooxidative damage and cell death in leaves corresponded to a different mechanism. Another factor that could possibly modulate the responses to $^1O_2$ in *flu* versus the wild type and *ch1* is the time scale: in the *flu* mutant, $^1O_2$ concentration rises immediately after the dark-to-light shift (within minutes or less), and this probably occurs transiently since this phenomenon depends on preaccumulation of the photosensitizer protochlorophyllide in darkness. In contrast, the $^1O_2$ level is likely to rise more gradually in *ch1* leaves with a production lasting as long as the light stress is maintained (and the PSII reaction centers are not severely damaged), leading to a more progressive $^1O_2$ stress. Independently of the factors underlying the involvement of EX in plant responses to $^1O_2$, the results of this study demonstrate that high-light-induced cell death is predominantly mediated by OXI1, not EX, in wild-type Arabidopsis leaves (Figs. 2–4).

The OXI1-Dependent $^1O_2$ Signaling Pathway Operates through a Different Route from the H_2O_2 Signaling Pathway

OXI1 belongs to the AGC kinase proteins, which constitute a large family of kinase proteins both in animals and plants (Pearce et al., 2010). The AGC kinase family (named after cAMP-dependent protein kinase A [PKA], cGMP-dependent protein kinase G [PKG], and phospholipid-dependent protein kinase C [PKC]) is a group of Ser/Thr protein kinases that share sequence similarities in their catalytic kinase domains with PKA, PKG, and PKC (Pearce et al., 2010) and are conserved among eukaryotic genomes. In mammalian systems, AGC kinases are important regulators of cell growth, metabolism, and cell death (Pearce et al., 2010). Although the catalytic kinase domains of plant AGC kinases share sequence similarities with those of mammalian AGC kinases, their molecular functions and targets are largely unknown (Garcia et al., 2012). In the Arabidopsis genome, there are 39 AGC kinase members (Bögre et al., 2003). To date, in different model plant species, several AGC kinases have been implicated in various developmental and physiological processes, including growth, morphogenesis, defense, and symbiosis (Rentel et al., 2004; Abuqamar et al., 2008; Fu et al., 2009; Garcia et al., 2012; Rademacher and Offringa, 2012; Zhu et al., 2015). In Arabidopsis, OXI1 is required for immunity against the pathogenic oomycete *Hyaloperonospora arabidopsidis* and the bacterial biotrophic pathogen *Pseudomonas syringae* pv tomato DC3000 (Rentel et al., 2004; Petersen et al., 2009). The rice (*Oryza sativa*) AGC kinase OsOXI1, an ortholog of the Arabidopsis OXI1, phosphorylates the residue Thr-233 of OsPTI-1a and then releases OsPTI-1a-mediated inhibition of disease resistance, resulting in positive contribution to basal resistance to the blast fungus *Magnaporthe oryzae* pv *oryzae* (Matsui et al., 2010). How the ROS signal is perceived in those responses is currently unknown. However, a signal transduction cascade, including the activation of protein kinases and downstream transcription factors, has been proposed in Arabidopsis as a mechanism of ROS sensing (Mittler et al., 2004; Van Breusegem et al., 2008; Meng and Zhang, 2013). Accordingly, the Arabidopsis AGC kinase, OXI1, is required for the full activation of MAPK3 and MAPK6 under H_2O_2 stress (Rentel et al., 2004). Clearly, this is not the case under $^1O_2$ stress: exposing the $^1O_2$-overproducing *ch1* mutant to high-light stress did not induce the expression of *MAPK3* and *MAPK6*, and this insensitivity to $^1O_2$ was confirmed in the double mutant *ch1*oxi1* (Fig. 5). Another remarkable difference was found in the regulation of *PTI1-1* and *PTI1-4*. In Arabidopsis and rice, Ser/Thr kinases of the PTI1 family were identified as interacting partners and kinase targets of OXI1, making them downstream components of the OXI1-dependent signaling pathway (Anthony et al., 2006; Matsui et al., 2010; Forzani et al., 2011). Arabidopsis PTI1-4 was recently shown to form protein complexes with MAPK3 and MAPK6 and could therefore mediate OXI1 regulation of the MAPKs (Forzani et al., 2011). However, the *PTI1-1* and *PTI1-4* genes were up-regulated by H_2O_2, but not by $^1O_2$ (Fig. 5). Based on those observations, we can conclude that the OXI1-dependent signaling pathway of $^1O_2$ is distinct from the OXI1-dependent immune responses to pathogens.

Interestingly, many AGC kinases contain phospholipid-binding domains (Pearce et al., 2010). In Arabidopsis plants exposed to pathogens, OXI1 is regulated by PDK1, and PDK1 is able to bind various lipids in vitro, resulting in a stimulation of its activity (Anthony et al.,

2004). As mentioned above, $^1O_2$ oxidation of the carotenoid β-carotene in PSII generates signal molecules involved in gene regulations mediated by $^1O_2$. It is also known that, because of their high unsaturation levels, lipids in plant leaves are sensitive to oxidation, with lipid peroxidation being usually a primary event associated with photooxidative stress (Mène-Saffrané et al., 2009). Oxidation of lipids can produce a variety of reactive derivatives, such as α,β-unsaturated aldehydes, with strong electrophilic properties (Farmer and Mueller, 2013). Those lipophilic reactive electrophilic species (RES) can function as secondary messengers in signal transduction and activation of redox sensitive transcription factors (Farmer and Davoine, 2007; Mueller and Berger, 2009; Farmer and Mueller, 2013). In the green microalga *Chlamydomonas*, the promoter of many $^1O_2$-responsive genes contains an electrophile response element that is required for their induction by lipid RES (Fischer et al., 2012). Whether reactive lipid derivatives can bind to plant AGC kinases is unknown. Nevertheless, considering that lipid RES can induce PCD (Farmer and Mueller, 2013) and the light stress conditions that induced PCD in wild-type and *ch1* Arabidopsis plants promoted lipid peroxidation (Figs. 2 and 3), this would be an attractive possibility for the regulation of the OXI-dependent pathway by high light and would deserve to be investigated in the future. However, OXI1 was not found to be localized in plastids (Anthony et al., 2004). Moreover, it has no N-terminal transit peptide, although this is not an unambiguous parameter since there are chloroplast proteins without cleavable transit peptides (Armbruster et al., 2009). The cytosolic localization of OXI1 could limit its interaction with chloroplast-generated lipid products.

Links between OXI1 and Oxylipins

In both *flu* and *ch1* mutants, several genes that encode proteins involved in the biosynthesis or signaling of oxylipins are up-regulated after the release of $^1O_2$ (Przybyla et al., 2008; Ramel et al., 2013a; Laloi and Havaux, 2015). Moreover, those gene expression changes associated with $^1O_2$-induced cell death are correlated with changes in oxylipin concentrations, suggesting that this type of compound could be involved in triggering cell death. In this work, high-light-stress-induced cell death in wild-type Arabidopsis was also associated with an accumulation of jasmonate and its precursor: the concentrations of jasmonate, JA-Ile, and OPDA in light-stressed leaves (Fig. 7A) were noticeably higher than the control concentrations (0.12, 0.07, and 0.23 ng mg^{-1} fresh weight, respectively). The role of oxylipins in $^1O_2$-mediated PCD was previously investigated by crossing the *flu* and *ch1* mutants with jasmonate-depleted mutants (Danon et al., 2005; Ramel et al., 2013a, 2013b). The analysis of cell death in the double mutant lines, in combination with or without the addition of exogenous jasmonate, revealed that jasmonate promotes the $^1O_2$-mediated cell death reaction, whereas OPDA seems to antagonize this effect. Our transcriptomic analysis of the effects of high-light stress on the *oxi1* Arabidopsis mutant indicated a down-regulation of genes of the jasmonate pathway. This effect was substantiated by the quantification of jasmonates in light-stressed leaves, which showed a substantial reduction of jasmonate and its active form, JA-Ile, in *oxi1* relative to the wild type (Fig. 7A). In addition, the microarray-based transcriptomic analysis revealed a down-regulation of ethylene biosynthesis and response genes, auxin-responsive genes, and gibberellin-mediated signaling genes in the *oxi1* mutant relative to the wild type, whereas genes that respond to salicylic acid were predominantly up-regulated (Supplemental Table S3). Salicylic acid is known to enhance tolerance in plants exposed to abiotic stresses (Mateo et al., 2006; Khan et al., 2015), while ethylene has been shown to function in synergy with jasmonic acid in regulation of stress tolerance (Xu et al., 1994; Lorenzo et al., 2003). Gibberellins have also been shown to play a role in regulation of tolerance to abiotic stresses, with increased gibberellins levels leading to increased susceptibility to these stresses (Colebrook et al., 2014). In addition, jasmonic acid activates the expression of auxin biosynthetic/responsive genes and vice versa (Dombrecht et al., 2007; Tiryaki and Staswick, 2002), implying a synergistic role of these hormones. Thus, the down-regulation of jasmonic acid-, gibberellin-, and ethylene-related genes coupled with the up-regulation of salicylic acid responsive genes may account for the enhanced tolerance of *oxi1* to high-light stress relative to the wild type.

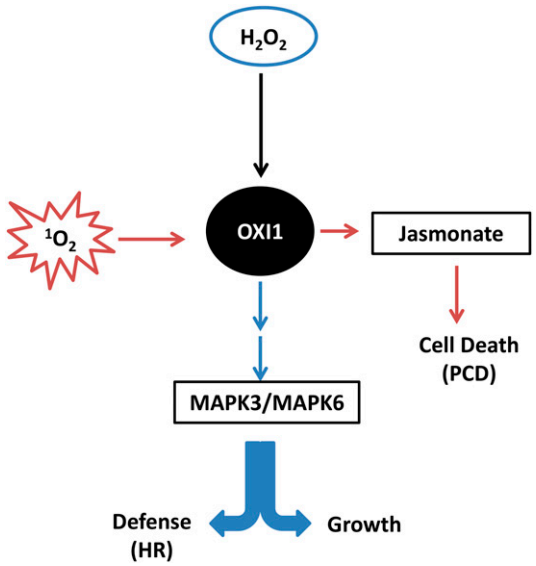

Figure 8. Proposed OXI1-dependent signaling pathways in plants after stimulation with $^1O_2$ stress produced predominantly during high-light stress (red arrows) and H_2O_2 stress (blue arrows). PCD triggered by $^1O_2$ is possibly regulated downstream of OXI1 by jasmonic acid, whereas during the hypersensitivity reaction (HR) triggered by H_2O_2, cell death is regulated by MAPK3 and MAPK6 downstream of OXI1.

Because jasmonate is a cell-death-promoting hormone in the presence of $^1O_2$ (Ramel et al., 2013b), the decreased levels of jasmonate and JA-Ile in *oxi1* mutant plants could participate in the phototolerance of this mutant. This idea is also supported by the finding that exogenous methyl-jasmonate cancelled the tolerance to cell death in the *oxi1* and *ch1*oxi1* mutants relative to the wild type and *ch1*, respectively, suggesting that jasmonate acts downstream of OXI1. Moreover, the transcriptome comparison between *oxi1* and the wild type indicated an up-regulation of genes coding for JAZ proteins in the *oxi1* mutant, which are negative regulator of jasmonate-induced transcriptomic modifications (Chini et al., 2007). This suggests that the *oxi1* mutation could also modify jasmonate signaling processes. Interestingly, high-light induction of genes encoding negative regulators of PCD was also noticeably stimulated in the *oxi1* mutants compared with the wild type (Fig. 6D). Thus, effects on jasmonate levels, on the abundance of negative regulators of jasmonate signaling, and on the expression levels of defense genes against PCD could contribute to the regulation of cell death by OXI1. Figure 8 summarizes the role of OXI1 as a central hub for ROS signaling where the H_2O_2 and $^1O_2$ signaling pathways intersect.

CONCLUSION

Data on $^1O_2$-induced PCD in wild-type Arabidopsis or in other plant species are scarce; most information on the $^1O_2$ intracellular signaling in vascular plants is derived from studies on the $^1O_2$-overproducing Arabidopsis mutants *flu* and *ch1*. Compared with those mutants, the responses to high-light stress are possibly different in the wild type since several ROS can be produced simultaneously in the chloroplasts and several superimposing signaling pathways can be expected. Nevertheless, using a biochemical approach based on $^1O_2$-specific lipid oxidation markers, Triantaphylidès et al. (2008) found that photodamage to Arabidopsis leaves is always associated with $^1O_2$-induced lipid peroxidation, indicating a central role for this high-energy form of oxygen in the execution of high-light-induced cell death in plants. Moreover, a good correlation was found between transcriptomes of numerous stresses, whether from light or dark treatments, and the transcriptome of the *flu* mutant (Mor et al., 2014), indicating that the role of $^1O_2$ in plant stress regulation and response could be ubiquitous and not necessary restricted to high-light stress. Here, based on a previous transcriptomic analysis of the *ch1* mutant exposed to different light conditions, we have identified a kinase that controls high-light-induced cell death in wild-type Arabidopsis. This regulator is part of a new signaling pathway, distinct from the H_2O_2-induced pathway and the EX-dependent $^1O_2$ signaling pathway, with a possible link with jasmonate signaling. Further studies will have to clarify this link and to characterize the molecular targets of the OXI1 kinase during $^1O_2$ stress.

MATERIALS AND METHODS

Plant Types, Growth Conditions, and Stress Treatments

Arabidopsis (*Arabidopsis thaliana*) lines used in this work were the wild-type ecotype Col-0, the $^1O_2$-overproducing *ch1* mutant (Ramel et al., 2013a), the *oxi1* null mutant (Gabi_355H08) deficient in the OXI1 kinase (Camehl et al., 2011), the *ch1*oxi1* double mutant, and the *ex1*ex2* double mutant deficient in the EX1 and EX2 proteins (Lee et al., 2007). Plants were grown for 5 to 8 weeks in short-day conditions (8 h/16 h, day/night) under a moderate PFD of 120 to 160 μmol photons m^{-2} s^{-1}, controlled temperature (20°C/18°C, day/night), and relative humidity (65%). Photooxidative stress was obtained by subjecting plants of the Col-0 background (wild type, *oxi1*, and *ex1*ex2*) to 1500 μmol m^{-2} s^{-1} PFD, 7°C/14°C day/night temperature, and 380 ppm CO_2 in a growth chamber. Because of their high photosensitivity (Ramel et al., 2013a), plants of the *ch1* background (*ch1* and *ch1*oxi1*) were subjected to less severe stress conditions: 1100 μmol photons m^{-2} s^{-1} PFD, 7°C/14°C temperature day/night, respectively, and 380 ppm CO_2. Photoacclimation in the *ch1* background was achieved by exposing plants to a moderate PFD of 450 μmol photons m^{-2} s^{-1} and day/night temperatures of 20°C/18°C for 2 d, as previously described (Ramel et al., 2013a).

Lipid Peroxidation Quantification and Imaging

Lipids were extracted from ~ 0.5 g of leaves frozen in liquid nitrogen. The leaves were ground in an equivolume methanol/chloroform solution containing 5 mM triphenyl phosphine, 1 mM 2,6-*tert*-butyl-*p*-cresol (5 mL g^{-1} fresh weight), and citric acid (2.5 mL g^{-1} fresh weight), using an Ultra-Turrax blender. Internal standard 15-HEDE was added to a final concentration 100 nmol g^{-1} fresh weight and mixed properly. After centrifugation at 700 rpm and 4°C for 5 min, the lower organic phase was carefully taken out with the help of a glass syringe into a 15-mL glass tube. The syringe was rinsed with ~ 2.5 mL of chloroform and transferred back into the tube. The process was repeated, and the lower layer was again collected and pooled to the first collection. The solvent was evaporated under N_2 gas at 40°C. The residues were recovered in 1.25 mL of absolute ethanol and 1.25 mL of 3.5 N NaOH and hydrolyzed at 80°C for 30 min. The ethanol was evaporated under N_2 gas at 40°C for ~ 10 min. After cooling to room temperature, pH was adjusted to 4 to 5 with 2.1 mL of citric acid. Hydroxy fatty acids were extracted with hexane/ether 50/50 (v/v). The organic phase was analyzed by straight phase HPLC-UV, as previously described (Montillet et al., 2004). HOTE isomers (9-, 12-, 13-, and 16-HOTE derived from the oxidation of the main fatty acid in Arabidopsis leaves, linolenic acid) were quantified based on the 15-HEDE internal standard.

Lipid peroxidation was also visualized in whole plants by autoluminescence imaging. Stressed plants were dark adapted for 2 h, and the luminescence emitted from the spontaneous decomposition of lipid peroxides was captured by a highly sensitive liquid N_2-cooled CCD camera, as previously described (Birtic et al., 2011). The images were treated using Image J software (National Institutes of Health).

PSII Photochemical Activity

The maximum quantum yield of PSII was determined by the chlorophyll fluorescence F_v/F_m ratio, measured in dark-adapted attached leaves using a PAM-2000 fluorometer (Walz), as previously described (Ramel et al., 2012a). The variable fluorescence F_v was calculated as the difference between maximum fluorescence F_m (obtained with an 800-ms pulse of intense white light) and the initial level fluorescence F_o (obtained with a 2-s pulse of far-red light).

Detection of $^1O_2$ Production

Production of $^1O_2$ was measured in attached leaves by fluorescence of the SOSG fluorescent probe (Invitrogen), as described previously (Ramel et al., 2012a). With the help of a 1-mL syringe (without the needle), 100 μM SOSG was pressure infiltrated into the leaves through the lower surface. Plants with SOSG-infiltrated leaves were exposed to a PFD of 1400 μmol m^{-2} s^{-1} at 7°C for 30 min. As a control treatment, plants with SOSG-infiltrated leaves were placed in the dark at room temperature for 30 min. SOSG fluorescence was then measured from leaf discs punched from the SOSG-infiltrated leaves using a fiberoptics-equipped Perkin-Elmer spectrofluorometer (LS 50B) with a 475-nm excitation light. SOSG fluorescence at 524 nm was normalized to fluorescence of chlorophyll *a* (at 647 nm) for each leaf disc.

Cell Death Determination

Cell death was measured in detached leaves of stressed plants by Evans blue staining as described by Shi et al. (2007) with slight modifications. Fourth to sixth rosette leaves were vacuum infiltrated with 0.1% (w/v) Evans blue for 40 min and then maintained under vacuum for 14 to 15 h. Leaves were thoroughly rinsed with water to remove unbound dye. Dye bound to dead cells was solubilized in 50% (v/v) methanol/H_2O containing 1% SDS for 14 to 15 h. Cell death was then estimated spectroscopically by measuring the absorbance ratio A_{600}/A_{680} of the resulting dye solution.

RNA Isolation and qRT-PCR

Total RNA was isolated from 100 mg of leaves using the Nucleospin RNA plant kit (Macherey-Nagel). The concentration was measured on a NanoDrop2000 (Thermo Scientific). First-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript reverse transcriptase kit (Takara). qRT-PCR was performed on a Lightcycler 480 real-time PCR system (Roche). Three microliters of a reaction mixture comprising SYBR Green I Master (Roche), 10 μM forward and reverse primers, and water was added to 2 μL of 10-fold diluted cDNA sample in a 384-well plate. The PCR program used was as follows: 95°C for 10 min, then 45 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 15 s. At least three biological replicates were performed for each gene tested. Primers for genes examined (Supplemental Table S4) were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

Microarray Transcriptomic Analysis

Transcript analysis was performed on a CATMAv6.2 (Complete Arabidopsis Transcriptome Microarray) system at the POPS Transcriptomic Platform of the Institute of Plant Sciences Paris-Saclay, France. A single high-density CATMAv6.2 microarray slide contains 12 chambers, each containing 219,684 primers representing all the Arabidopsis genes: 30,834 probes corresponding to CDS TAIRv8 annotation (including 476 probes of mitochondrial and chloroplast genes) + 1289 probes corresponding to EUGENE software predictions. Moreover, it included 5352 probes corresponding to repeat elements, 658 probes for miRNA/MIR, 342 probes for other RNAs (rRNA, tRNA, snRNA, soRNA), and, finally, 36 controls. Each long primer is triplicate in each chamber for robust analysis and in both strands. The transcriptome compared RNA from wild-type and *oxi1* plants under control growth conditions or after high-light and low-temperature stress (1500 μmol photon m^{-2} s^{-1} and 7°C/14°C day/night). RNA was extracted using the Nucleospin RNA plant kit (Macherey-Nagel). Synthesis of cDNA, amplification, labeling, hybridization, and scanning of slides were performed as described elsewhere (Lurin et al., 2004). Three different comparisons were performed: *oxi1*-control versus wild type-control, *oxi1* stress versus wild-type stress, and *oxi1* stress versus *oxi1* control. For each comparison, three biological replicates (RNA from three individual plants for each treatment) were performed. Two technical replicates were performed for each comparison, with a reversal of fluorochrome for each pool of RNA (one dye was swapped per comparison). The hybridization and washing were performed according to NimbleGen Arrays User's Guide v5.1 instructions. Two-micron scanning was performed with an InnoScan900 scanner (Innopsys), and raw data were extracted using Mapix software (Innopsys).

Experiments were designed with the statistics group of the IPS2. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). For each array, a global intensity-dependent normalization using the loess procedure (Yang et al., 2002) was performed to correct the dye bias. The differential analysis is based on the log ratios averaging over the duplicate probes and over the technical replicates. Hence, the number of available data for each gene equals the number of biological replicates and is used to calculate the moderated t test (Smyth, 2004). Under the null hypothesis, no evidence that the specific variances vary between probes is highlighted by Limma, and, consequently, the moderated t statistic is assumed to follow a standard normal distribution. To control the false discovery rate, adjusted P values found using the optimized FDR approach of Storey and Tibshirani (2003) were calculated. We considered as being differentially expressed the probes with an adjusted P value ≤ 0.05. Analysis was done with the R software. The function SqueezeVar of the library limma has been used to smooth the specific variances by computing empirical Bayes posterior means. The library kerfdr has been used to calculate the adjusted P values.

Functional classification and analysis of the differentially expressed genes were performed using the DAVID functional classification tool (Huang et al., 2009a, 2009b). Enrichment clusters/terms with EASE score (P value) < 0.05 were considered as significantly different.

Jasmonate Treatment

Wild-type and *oxi1* mutant plants were subjected to high-light stress (1500 μmol $m^{-2}s^{-1}$ PFD and 7°C/14°C day/night temperature) and simultaneously sprayed twice a day with a solution of 1 mM methyl-jasmonate in 25 mM phosphate buffer and 0.1% Tween 20 ($\sim$ 400 μL per plant), as previously described (Ramel et al., 2013a). Similar treatment was performed on *ch1* and *ch1*oxi1* plants at PFD of 1100 μmol m^{-2} s^{-1}. After $\sim$ 48 h of stress, the effect of jasmonate on *oxi1* and *ch1*oxi1* plants was estimated relative to wild-type and *ch1* plants, respectively, by autoluminescence imaging and HPLC quantification of HOTE.

Jasmonate Quantification

Jasmonic acid, its active form JA-Ile, and its precursor OPDA were quantified by UPLC-MS as described by Stingl et al. (2013), with some modifications. Frozen leaves from high-light-stressed wild-type and *oxi1* plants were ground under liquid N_2, and 100 mg of powder was used for extraction in a 1.5-mL screw cap tube. Extraction solution, ethylacetate/formic acid (99/1), internal standard jasmonyl nor-Val, and a bead were added to the ground leaf tissue and agitated for 3 min at 20 Hz. After centrifugation, the supernatant was evaporated under N_2 gas. The residue was reconstituted in 100 μL of acetonitrile/ water (1/1). Aliquots were then analyzed by UPLC-MS on a C8 Kinetex column (Phenomenex) of dimension 150 mm $\times$ 2.1 mm $\times$ 1.7 μm, maintained at 45°C. Elution was achieved by gradually increasing gradient of solvent B (90% methanol/10% isopropanol) from 37% to 97% relative to solvent A (90% water/10% methanol) at a rate of 0.3 mL min^{-1}.

Accession Numbers

Sequence data from this article can be found in The Arabidopsis Information Resource or GenBank/EMBL database under the following accession numbers (in parentheses): OXI1 (At3g25250), EX1 (At4g33630), EX2 (At1g27510), and CH1 (At1g44446).

Microarray data from this article were deposited at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, accession no. GSE61478) and at CATdb (http://urgv.evry.inra.fr/CATdb/; Project CEA13-01_oxi1) according to the "Minimum Information About a Microarray Experiment" standards.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effect of visible light on the fluorescence of a SOSG solution.

Supplemental Figure S2. OXI1-dependent pathway in plants during pathogen attack.

Supplemental Table S1. Functional classification list of induced and repressed genes in the comparison *oxi1*-control versus wild type-control.

Supplemental Table S2. Functional classification list of induced and repressed genes in the comparison *oxi1*-stress versus *oxi1*-control.

Supplemental Table S3. Functional classification list of induced and repressed genes in the comparison *oxi1*-stress versus wild type-stress.

Supplemental Table S4. Primer sequences used in qRT-PCR transcript profiling.

ACKNOWLEDGMENTS

We thank Christophe Laloi (Aix Marseille University) for providing *ex1*ex2* double mutant seeds and for critical reading of the article. The *oxi1* single mutant was a kind gift from Ana Victoria Garcia and Heribert Hirt (URGV, INRA Evry). We would like to thank Martin Mueller (Wuerzburg University) for

providing standards for jasmonate quantification, and Philippe Ortet (CEA) for his help in analyzing the transcriptomic data. We also acknowledge the support of the Phytotec platform and the HelioBiotec platform funded by the European Union (FEDER), the Région Provence Alpes Côte d'Azur, the French Ministry of Research, and CEA.

Received October 5, 2015; accepted January 7, 2016; published January 8, 2016.

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