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Roles of Defense Hormones in the Regulation of Ozone-Induced Changes in Gene Expression and Cell Death

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ABSTRACT

Apoplast, the diffusional space between plant cell plasma membranes, is an important medium for signaling within and between the cells. Apoplastic reactive oxygen species (ROS) are crucial signaling molecules in various biological processes. ROS signaling is interconnected with the response to several hormones, including jasmonic acid (JA), salicylic acid (SA) and ethylene. Using ozone (O_3) to activate apoplastic ROS signaling, we performed global and targeted analysis of transcriptional changes and cell death assays to dissect the contribution of hormone signaling and various transcription factors (TFs) in the regulation of gene expression and cell death. The contributions of SA, JA, and ethylene were assessed through analysis of single, double, and triple mutants deficient in biosynthesis or signaling for all three hormones. Even in the triple mutant, the global gene expression responses to O_3 were mostly similar to the wild-type. Cell death in the JA receptor mutant *coi1-16* was suppressed by impairment of the NADPH oxidase RBOHF, suggesting a role for a ROS signal in limiting the spread of cell death. In response to apoplastic ROS, there is not a single signaling pathway that regulates gene expression or cell death. Instead, several pathways regulate the apoplastic ROS response via combinatorial or overlapping mechanisms.

Keywords: cell death, ethylene, gene expression, jasmonic acid, reactive oxygen species, salicylic acid

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INTRODUCTION

Plants use reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2), as signaling molecules in the regulation of stress responses, development, and cell death (Jaspers and Kangasjärvi, 2010; Suzuki et al., 2011; Considine and Foyer, 2014). Efficient signaling requires spatiotemporal control of the production and accumulation of these ROS. Plants produce ROS in different tissues as well as in specific subcellular compartments during metabolism, photosynthesis, and photorespiration (Vaahtera et al., 2014). In addition, controlled ROS production in plants is mediated by several enzymes including peroxidases and NADPH oxidases (also known as respiratory burst oxidase homologs [RBOHs]). Depending on the type of ROS produced (singlet oxygen, O_2^- or H_2O_2) and where it is produced (apoplast, chloroplast, mitochondria, or other locations) different signaling pathways are activated (Gadjev et al., 2006; Vaahtera et al., 2014). Hence, plants must have mechanisms to precisely determine where the ROS are produced and to activate appropriate downstream responses (Wrzaczek et al., 2013).

Apoplastic ROS signaling is an essential component of cell-to-cell signaling. A ROS signal produced by RBOHD is rapidly relayed throughout the plant in response to several stresses (Miller et al., 2009). Both RBOHD and RBOHF are also important for ROS production and defenses in response to biotic and abiotic stresses (Marino et al., 2012; Kadota et al., 2014). In addition, a peroxidase-dependent apoplastic ROS burst is an essential part of pattern-triggered immunity (Daudi et al., 2012; O'Brien et al., 2012). Clearly, apoplastic ROS have an important signaling role in stress responses; however, little is known how ROS are perceived and how the downstream signaling is activated. One experimental complication in the

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study of apoplastic ROS are other biological effects elicited by the treatment used to activate the ROS production. For example, if pathogen infection is used to activate RBOH-mediated ROS production, there are also other signaling pathways activated in the pathogen infection, such as PAMP (Pathogen-associated Molecular Pattern) signaling (Bernoux et al., 2011; Macho and Zipfel, 2014). Thus, a clean system to deliver apoplastic ROS would be required to study the role of ROS separate from concurrent activation of other signaling pathways.

Ozone (O_3), a gaseous ROS, has been used extensively as a tool to study apoplastic ROS signaling and its role in cell death, defense signaling, and regulation of gene expression (Vainonen and Kangasjärvi, 2014). In response to O_3, there is a rapid activation of Ca^{2+} signaling (Short et al., 2012), MPK signaling (Ahlfors et al., 2004), and changes in gene expression (Mahalingam et al., 2006; Blomster et al., 2011). Sensitive plant species exposed to O_3 develop lesions from the activation of cell death programs (Overmyer et al., 2005; Brosché et al., 2010). Importantly, O_3 treatment can trigger the plants' own apoplastic ROS production machinery, thus making O_3 a highly useful tool for the study of apoplastic ROS signaling (Wohlgemuth et al., 2002).

Studies on signaling events downstream of apoplastic ROS have focused on the role of stress hormones, especially salicylic acid (SA), jasmonic acid (JA), and ethylene, in the regulation of cell death and gene expression (Rao and Davis, 1999; Rao et al., 2002; Overmyer et al., 2003; Tuominen et al., 2004; Overmyer et al., 2005). During the activation of O_3-induced cell death, ethylene promotes cell death while JA has a protective role. The role of SA is more complicated; depending on the genetic background, it can have either cell death-promoting or protective roles (Rao and Davis, 1999; Overmyer et al., 2005; Xu et al., 2014). *Arabidopsis thaliana* offers a convenient system to study apoplastic ROS signaling due to the availability of hormone signaling and biosynthesis mutants including the JA receptor mutant *coi1* (CORONATINE INSENSITIVE1), the essential ethylene signaling mutant *ein2* (ETHYLENE INSENSITIVE2), and the SA biosynthesis mutant *sid2* (SALICYLIC ACID INDUCTION DEFICIENT2 also known as ISOCHORISMATE SYNTHASE1). The three hormones have both synergistic and antagonistic interactions, where the suppression of JA signaling by SA is the most studied response after pathogen infection or insect attack (Pieterse et al., 2012). This suppression can be counteracted by pre-activation of ethylene signaling (Leon-Reyes et al., 2010). The contribution and interaction between all three hormones was quantified after *Pseudomonas syringae* infection using all possible mutant combinations between a JA biosynthesis mutant *dde2* (*delayed dehiscence2*), also known as *aos* (ALLENE OXIDE SYNTHASE), *ein2*, and *sid2* (Tsuda et al., 2009; Kim et al., 2014). This revealed that all three hormones contribute positively to pathogen resistance and that the main inhibitory interaction was ethylene inhibition of JA signaling. *Arabidopsis* mutants defective in JA biosynthesis or signaling develop cell death in response to O_3, which can be suppressed by simultaneous inactivation of ethylene signaling (Tuominen et al., 2004). Abscisic acid (ABA) is an important hormone in defenses against abiotic stresses including drought and cold. ABA also interacts at various steps of SA, JA, and ethylene signaling; ABA is antagonistic to SA in pathogen response and ABA treatment reduces the expression of JA and ethylene responsive defense genes (Anderson et al., 2004; Pieterse et al., 2012).

Apart from cell death, apoplastic ROS signaling also triggers broad transcriptional responses, where the expression of thousands of genes is altered (Blomster et al., 2011; Brosché et al., 2014). Very few transcription factors (TFs) mediating this response have been identified to date. Analysis of the promoter from *NUDT7* (*NUDIX HYDROLASE 7*) in two different *Arabidopsis* accessions, Col-0 and Ws, revealed that only the promoter in the Ws accession was O_3-inducible, which was caused by a 16-bp insertion containing a GCC-box (Muthuramalingam et al., 2015). The ERF1 (ETHYLENE RESPONSE FACTOR 1) TF bound to the GCC-box in the Ws *NUDT7* promoter (Muthuramalingam et al., 2015). Additional TFs possibly involved in the regulation of apoplastic ROS signaling were identified through promoter enrichment in O_3-regulated genes and include WRKY, TGA, and ABF TFs (Blomster et al., 2011). For H_2O_2, some TFs regulating gene expression have been identified, including ERF6 (Wang et al., 2013), ANAC013 (De Clercq et al., 2013), and ANAC017 (Ng et al., 2013). However, given the large amount of apoplastic ROS-regulated genes, there are likely additional TFs regulating apoplastic ROS signaling.

Here we used single, double, and triple mutants in transcriptome analysis and cell death assays to quantify the contribution of hormone signaling in relation to apoplastic ROS signaling. Furthermore, we identified TGA TFs involved in ROS-regulated gene expression and we dissected components involved in JA-associated cell death. In particular, we show that signaling via SA, JA, and ethylene contributes to about 30% of apoplastic ROS-regulated changes in gene expression. Hence, both hormone-dependent and -independent signaling pathways mediate apoplastic ROS signaling.

RESULTS AND DISCUSSION

The Transcriptome of the Triple Mutant *coi1 ein2 sid2*

SA, JA, and ethylene have both synergistic and antagonistic interactions (Tuominen et al., 2004; Pieterse et al., 2012). To make a mutant background that would be deficient in signaling for all three hormones, we generated the *coi1-16 ein2-1 sid2-1* triple mutant and the corresponding double mutants. The *coi1-16* mutant was identified in the *gl1* (*glabra1*) background and has a second site mutation in *pen2* (*penetration2*), which could lead to altered defense responses (Westphal et al., 2008). Before we used *coi1-16* in crosses, the *pen2* and *gl1* mutations were removed through backcrossing with Col-0 (data not shown).

The transcriptome of *coi1 ein2 sid2* and Col-0 was obtained via RNA sequencing (RNA-seq) in control and 2-h O_3 treatment (350 nl l^{-1}) using three biological repeats (Supplemental Table 1). At this time point, the largest number of O_3-regulated changes in gene expression have been detected (Blomster et al., 2011); and the 2-h time point is better correlated with the regulation of cell death than other time points (Brosché et al., 2014). First, the different biological repeats were analyzed with