An NADPH-Oxidase/Polyamine Oxidase Feedback Loop Controls Oxidative Burst Under Salinity

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The apoplastic polyamine oxidase (PAO) catalyzes the oxidation of the higher polyamines spermidine and spermine, contributing to hydrogen peroxide (H$_2$O$_2$) accumulation. However, it is yet unclear whether apoplastic PAO is part of a network that coordinates the accumulation of reactive oxygen species (ROS) under salinity or if it acts independently. Here, we unravel that NADPH oxidase and apoplastic PAO cooperate to control the accumulation of H$_2$O$_2$ and superoxides (O$_2^-$) in tobacco (Nicotiana tabacum). To examine to what extent apoplastic PAO constitutes part of a ROS-generating network, we examined ROS accumulation in guard cells of plants overexpressing or down-regulating apoplastic PAO (lines S2.2 and A2, respectively) or down-regulating NADPH oxidase (line AS-NtRbohD/F). The H$_2$O$_2$-specific probe benzene sulfonyl-H$_2$O$_2$ showed that, under salinity, H$_2$O$_2$ increased in S2.2 and decreased in A2 compared with the wild type. Surprisingly, the O$_2^-$-specific probe benzene sulfonyl-SO$_2$ showed that O$_2^-$ levels correlated positively with that of apoplastic PAO (i.e. showed high and low levels in S2.2 and A2, respectively). By using AS-NtRbohD/F lines and a pharmacological approach, we could show that H$_2$O$_2$ and O$_2^-$ accumulation at the onset of salinity stress was dependent on NADPH oxidase, indicating that NADPH oxidase is upstream of apoplastic PAO. Our results suggest that NADPH oxidase and the apoplastic PAO form a feed-forward ROS amplification loop, which impinges on oxidative state and culminates in the execution of programmed cell death. We propose that the PAO/NADPH oxidase loop is a central hub in the plethora of responses controlling salt stress tolerance, with potential functions extending beyond stress tolerance.

Several enzymatic and nonenzymatic reactions control the production of reactive oxygen species (ROS; Gilroy et al., 2014; Foyer and Noctor, 2016). Superoxide ions (O$_2^-$) are generated mainly by the respiratory burst oxidase homologs NADPH oxidases (encoded by the Rboh genes), and O$_2^-$ dismutation by superoxide dismutase is considered one of the major routes for subsequent hydrogen peroxide (H$_2$O$_2$) production (Torres et al., 2002; Kwak et al., 2003; Wang et al., 2013; Baxter et al., 2014).

The homeostasis of ROS is controlled by low-M$_2$ intermolecular and intramolecular compounds, such as the polyamines (PAs). PAs are highly reactive aliphatic polycations; the main PAs in plants are the diamine putrescine (Put) and the so-called higher PAs, spermidine (Spd; triamine) and spermine (Spm; tetramine; Tiburcio et al., 2014; Saha et al., 2015, and refs. therein). PA homeostasis affects a vast range of dynamic developmental and metabolic processes (Paschalidis and Roubelakis-Angelakis, 2005a, 2005b; Moschou et al., 2009; Wu et al., 2010; Moschou and Roubelakis-Angelakis, 2014; Tiburcio et al., 2014; Pal et al., 2015). The oxidation of PAs is catalyzed by amine oxidases (AOs). AOs, such as the diamine oxidases (DAOs; or copper-containing AOs) and the flavin-containing polyamine oxidases (PAOs), localize either intercellularly (i.e. apoplast) or intracellularly (i.e. cytoplasm and peroxisomes). DAOs oxidize mainly Put, but also Spd and Spm (with much lower efficiency), yielding H$_2$O$_2$ and aminoaldehydes. The apoplastic PAOs terminate oxidize Spd and Spm, yielding aminoaldehydes and H$_2$O$_2$, while the intracellular PAOs (also referred to as...
back-converting PAOs) oxidize PAs to produce H$_2$O$_2$, an aminoaldehyde, and a PA with one less amino group (in the order tetramine→triamine→diamine; Angelini et al., 2010; Pottosin and Shabala, 2014). Through their catabolic oxidative deamination, PAs increase the intracellular and extracellular H$_2$O$_2$ load.

Under physiological or stress conditions, the rate of ROS generation/scavenging determines their steady level; this rate is integrated into a multitude of vital signaling cues. ROS seem to be multifaceted players: at low levels, they are efficiently scavenged by enzymatic and nonenzymatic antioxidants, present in nearly all cellular compartments (Mittler et al., 2004; Miller et al., 2010; Suzuki and Mittler, 2012; Baxter et al., 2014; Foyer and Noctor, 2015); at medium levels and up to a threshold signature, ROS participate in downstream signaling cascades that activate stress-protective effector genes/mechanisms. When a certain high level is reached, oxidative stress is established and ROS participate in a plethora of destructive pathways that culminate in the induction of programmed cell death (PCD; Moschou et al., 2008a, 2008b; Gémes et al., 2011; Moschou and Roubelakis-Angelakis, 2014).

PAOs and NADPH oxidases, major ROS generators, have been mostly studied separately, and it remains unknown whether they are functionally linked. Their involvement in similar processes points at their possible interplay. Perhaps the best example of the convergent action of PAOs and NADPH oxidases is the control of stomatal aperture. In Arabidopsis (Arabidopsis thaliana) guard cells, aminic acid (ABA) induces the production of H$_2$O$_2$ arising from O$_2^-$ generated by NADPH oxidases. The produced H$_2$O$_2$ activates, among others, downstream ROS-dependent Ca$^{2+}$ channels contributing to cytosolic Ca$^{2+}$ increase (Kwak et al., 2003; Desikan et al., 2004; Baxter et al., 2014). Likewise, ABA induces the increase of H$_2$O$_2$ in the apoplast through the up-regulation of peroxidase and apoplastic PAO (Zhu et al., 2006).

In an attempt to increase our understanding of how PAOs can contribute to processes where NADPH oxidases are involved, we examined the interplay between these genes/enzymes. To this end, we used tobacco (Nicotiana tabacum) plants up-/down-regulating apoplastic PAO (lines S2.2 and A2, respectively; Moschou et al., 2008a, 2008b) and tobacco plants down-regulating two NADPH oxidase genes (AS-NtRbohD and AS-NtRbohF; Ji and Park, 2011). We used guard cells for real-time in vivo monitoring of apoplastic PAO/NADPH oxidase-derived H$_2$O$_2$ and O$_2^-$ intracellularly and intercellularly (Song et al., 2014). Our results provide evidence for an interplay of PAO/NADPH oxidase that culminates in the induction of programmed cell death.

**RESULTS**

**Apoplastic PAO Represents the Main Spd Oxidation Source**

Considering the large number of AOs in plants (Moschou et al., 2008c), we aimed at determining the relative contribution of apoplastic versus intracellular PA oxidation to H$_2$O$_2$ production during salinity. We previously established that, during salt stress, Spd is secreted into the apoplast, where it is oxidized by the apoplastic PAO (Moschou et al., 2008b). However, the contribution of intracellular AOs to Spd oxidation under the same conditions was not examined. In an attempt to dissect the contribution of different AOs to H$_2$O$_2$ production, we used tobacco transgenic lines overexpressing or down-regulating ZmPAO (ZmPAO [line S2.2] and AS-ZmPAO [line A2], respectively; Moschou et al., 2008a, 2008b). Line S2.2 shows increased while A2 shows reduced apoplastic PAO activity (Moschou et al., 2008b). In contrast to our previous works, here we used leaves that were not fully expanded, in order to take into consideration the importance of PAOs in developmental processes, such as leaf expansion during salt stress (Rodriguez et al., 2009). At this stage, the profile of PAs in the wild type, A2, and S2.2 was somewhat different from what has been described previously (Supplemental Fig. S1; Moschou et al., 2008a, 2008b). However, the observed expected increase of PAs in A2 and the decrease of higher PAs (Spd and Spm) in S2.2 suggest that the apoplastic PAO controls FA levels in expanding leaves, as was the case for the fully expanded ones (Moschou et al., 2008a, 2008b).

Next, we determined the total cellular capacity of Spd oxidation (terminal plus back conversion) versus terminal Spd oxidation in the wild type, A2, and S2.2. To achieve this, we developed an in-gel Spd oxidation assay that determines total Spd oxidation activity. We compared the results obtained from this in-gel Spd oxidation assay with those obtained from a colorimetric assay that determines terminal Spd oxidation (Supplemental Fig. S2A). The in-gel assay is based on the fact that H$_2$O$_2$ produced by Spd oxidation reacts with 3,3′-diaminobenzidine (DAB), forming a brownish adduct that denotes the gel regions (bands) enriched in Spd oxidation activity. In the wild type, Spd oxidase can be visualized as multiple bands (three main ones), with a major isoenzyme (greater than 50%) showing high mobility (referred to hereafter as anodal). This isoenzyme pattern is consistent with the large number of predicted PAOs and DAOs in the tobacco genome (at least one apoplastic and four intracellular PAOs and more than 12 DAOs; Supplemental File S1). However, we could not define a large number of bands in the wild type, suggesting that some isoenzymes may show similar mobility on the gel, preventing their separation, may not be present in leaves, or could be refractory to this analytical method. In A2, the major anodal Spd oxidase isoenzymes were depleted, suggesting that they most likely correspond to apoplastic PAO isoforms (Supplemental Fig. S2, A and B). In S2.2, we observed a significant increase of the in-gel Spd oxidase potential and, in particular, the appearance of an additional fast-migrating band that could not be seen in the wild type and A2. Although we could only achieve a fair resolution of isoenzymes, we assume that the fast-migrating...
band corresponds to the apoplastic maize (Zea mays) PAO isoenzyme (ZmPAO), which is overexpressed in S2.2 (predicted mass approximately 53 kD). We also observed in S2.2 an increase of additional bands, which were significantly less mobile than the band that presumably corresponds to maize PAO. These isoenzymes could correspond to a posttranslationally modified maize PAO or different maize PAO fractions (Cona et al., 2006). Alternatively, the increase in apoplastic PAO may signal the up-regulation of other AOs, or simply the DAB adduct, due to its higher production in S2.2, may diffuse, producing erroneous bands. The quantification of bands in the three genotypes showed that the overall Spd oxidase activity in S2.2 increased significantly by 2-fold, mostly due to the increase of the anodal isoenzymes; A2 lines showed a 2-fold decrease due to the absence of the major anodal isoenzyme. Taken together, these results suggest that the apoplastic PAO represents the major Spd oxidase activity.

In the colorimetric assay, DAO activity (terminal oxidation of Put) was not increased significantly among the three genotypes (Supplemental Fig. S2C). On the other hand, the terminal Spd oxidase activity (mainly apoplastic PAO) was reduced significantly in A2 lines, while in S2.2 it increased by 3-fold (Supplemental Fig. S2C). In addition, apoplastic PAO activity was highly responsive to 200 mM salt treatment (referred to hereafter as NaCl treatment), exhibiting a significant increase (Moschou et al., 2008b), whereas the cathodal total Spd oxidase activity responded moderately to NaCl treatment (Supplemental Fig. S2, B and C).

To further substantiate the previous finding, we examined the Spd oxidase activity of protoplasts by the colorimetric 4-aminopterine oxidation assay used to determine the activity of both terminal and back-converting PAOs and DAOs (Tavladoraki et al., 2006). The activity of Spd oxidase in wild-type protoplasts was negligible (close to background levels [as a positive control, purified AtPAO3 was used in these assays]; Moschou et al., 2008c), suggesting that the main Spd oxidase activity resides in the apoplastic compartment. Taken together, the data produced through the in-gel and in vitro assays suggest that the apoplastic PAO accounts for at least 50% of the total Spd oxidase activity in expanding tobacco leaves; therefore, it is the major Spd oxidase activity during salinity.

**Apoplastic PAO Impacts O$_2^\cdot$ Production**

Previously, we found that S2.2 plants show increased superoxide dismutase activity, suggesting that O$_2^\cdot$ homeostasis may be compromised in these plants (Moschou et al., 2008a). NaCl treatment can be used to examine the contribution of apoplastic PAO to H$_2$O$_2$ levels, and the in situ ROS detection assay is a powerful tool in the estimation of PAO-derived H$_2$O$_2$ levels (Moschou et al., 2008a, 2008b). Under control conditions, we could not detect significant differences in the staining intensities for O$_2^\cdot$ and H$_2$O$_2$ among the three genotypes (Fig. 1, A and B, 0 h). NaCl treatment induced the increase of both ROS in a time-dependent manner. One to 24 h posttreatment, A2 leaves contained lower, while S2.2 leaves contained higher, levels of O$_2^\cdot$ and H$_2$O$_2$ than the wild type (Fig. 1, A and B, 1 h). These results were confirmed using an in vitro quantification assay for H$_2$O$_2$ (Fig. 1C) and suggest that apoplastic PAO influences the production of not only H$_2$O$_2$ but also of O$_2^\cdot$ under stress conditions.

**The Apoplastic PAO-Dependent ROS Accumulation Is Sufficient to Induce PCD within the First Few Hours of NaCl Treatment**

We have shown that apoplastic PAO is critically required for PCD execution during prolonged NaCl stress (stress treatment in the range of several days; Moschou et al., 2008b). Here, we examined to what extent under short-term NaCl treatments (in the range of hours) apoplastic PAO-generated ROS are sufficient to induce PCD hallmarks. The array of events that precede PCD execution during NaCl stress are yet unclear and might be context/species specific. S2.2 showed an early accumulation of oxidized proteins (Supplemental Fig. S3, A and B; 1 h posttreatment), in contrast to A2. Significant accumulation of necrotic cells was observed 6 h posttreatment and onward (Supplemental Fig. S3C). Thus, the accumulation of oxidized proteins and ROS seems to precede PCD. Our results suggest that short NaCl treatments (i.e. less than 24 h) are enough to induce apoplastic PAO-derived ROS accumulation of sufficient amounts to induce PCD hallmarks. In addition, our results suggest that protein oxidation and the accumulation of ROS are upstream events in the execution of NaCl-induced PCD, at least under the described conditions.

**Guard Cells Reflect the Real-Time ROS Accumulation Post-NaCl Treatment**

Guard cells have been used to study real-time ROS accumulation (Song et al., 2014). In these cells, the NADPH oxidase genes RbohD and RbohF are involved in ABA-mediated stomatal closure (Zhang et al., 2001; Kwak et al., 2003; Song et al., 2014). Similarly, apoplastic PAO contributes to ABA-induced H$_2$O$_2$ production in maize under control conditions (Xue et al., 2008).

First, we used the unspecific ROS probe 2',7'-dichlorofluorescein diacetate (DCFDA) to determine ROS production in guard cells. DCFDA is hydrolyzed by cellular esterases to form DCFH, which is oxidized in the presence of peroxidases by hydroxyl or organic peroxyl radicals and the reactive nitrogen species NO$^\cdot$ and ONOO$^-$ to form the fluorescent dye dichlorofluorescein (DCF; Myhre et al., 2003). The intensity of DCF reflects the formation of general reactive species (RS; the sum of nitrogen and oxygen reactive species) rather than specific ones, providing a rough estimate of ROS production. In guard cells of the wild type, A2, and S2.2, the fluorescence of DCF coincided with the total H$_2$O$_2$ and O$_2^\cdot$ production determined using the in situ detection method (Fig. 2A). In particular, under control
conditions, no significant differences were observed in DCF fluorescence in guard cells among the three genotypes (Fig. 2, 0 h). Thus, under control conditions, apoplastic PAO does not seem to influence the RS levels. However, 1 and 6 h posttreatment, S2.2 contained higher, while A2 contained lower, DCF compared with the wild type (Fig. 2, 1 and 6 h). DCF accumulated mainly in the nucleus and chloroplasts, but also at the cell margins, of S2.2 guard cells (Fig. 2C, 6 h). This accumulation pattern does not necessarily reflect the RS production sites. In accordance, previous studies suggested that different ROS probes tend to accumulate to distinct intracellular sites that may not coincide with the ROS-producing sites (Snyrychova et al., 2009).

Next, we used more specific dyes to estimate H$_2$O$_2$ levels in guard cells. To this end, we evaluated two different sets of fluorescent probes. First, we used the H$_2$O$_2$ probes Amplex Red (AR) and Amplex Ultra Red (AUR; Ashtamker et al., 2007), which are used to estimate H$_2$O$_2$ levels intracellularly and extracellularly, respectively. Under control conditions, no significant differences could be observed among the three genotypes in AR and AUR fluorescence intensities (Supplemental Fig. S4, A and B, 0 h). One and 6 h posttreatment with NaCl, an increase in AR and AUR fluorescence was detected in all three genotypes, mostly in S2.2 plants (Supplemental Fig. S4A). Significant AR and AUR fluorescent signals accumulated in chloroplasts. A2 plants showed reduced AR and AUR fluorescence (6 h), preceded by a transient increase of AUR 1 h posttreatment. This transient increase may reflect the presence of high levels of peroxidase in the apoplast of A2 plants or the interference of the probe with a cellular metabolite. Snyrychova et al. (2009) showed that AR and AUR are highly sensitive to peroxidase levels, similar to DCF and DAB, which also are highly sensitive to peroxidase (Noctor et al., 2016).

Then, we employed a peroxidase-independent method for the estimation of H$_2$O$_2$ levels. We used the highly specific benzene sulfonyl (BES)-H$_2$O$_2$ and BES-H$_2$O$_2$-Ac probes to estimate intracellular/extracellular H$_2$O$_2$ levels, respectively (Fig. 3). This probe pair is converted to fluorescent molecules in the presence of esterases and might be more specific than AR and AUR, which are used more extensively in the in vitro determinations of H$_2$O$_2$ where peroxidases are added in surplus (Noctor et al., 2016). By using BES-H$_2$O$_2$ and BES-H$_2$O$_2$-Ac, we observed a similar trend of H$_2$O$_2$ accumulation in S2.2 (Fig. 3). However, in this case, we did not observe the transient increase of H$_2$O$_2$ in A2 1 h posttreatment (compare Fig. 3B with Supplemental Fig. S4B). Taken together, our results confirm that guard cells can be used efficiently to monitor real-time ROS accumulation. In addition, guard cells offer some unique advantages over other cell tissues/types for ROS detection. They are homogenous, readily accessible for microscopic observation, and they show a profound physiological responsiveness to short-term NaCl treatment. In addition, we confirm that BES-H$_2$O$_2$ and BES-H$_2$O$_2$-Ac are more specific probes for the detection of H$_2$O$_2$ levels in plants. Nevertheless, a careful assessment of different probes might be required depending on the context/tissue.

**PAO-Derived H$_2$O$_2$ Coincides with O$_2^-$ Production in Guard Cells**

Intracellular generation of O$_2^-$ was detected using BES-So-AM, a highly specific fluorescent probe for O$_2^-$.
Under control conditions, no significant accumulation of $O_2^-$ could be detected in the three genotypes (Fig. 4A, 0 h). One and 6 h post-treatment, the levels of intracellular $O_2^-$ were increased significantly in guard cells of wild-type and S2.2 plants compared with A2 plants (Fig. 4A, 1 and 6 h). Particularly, fluorescent BES-So-AM accumulated in the nucleus and chloroplasts of the wild type. BES-So-AM was also detected in cell margins of S2.2 guard cells. Thus, although the 1-h post-NaCl treatment pixel intensity of BES-So-AM fluorescence differed marginally between the wild type and S2.2, the difference in the total intracellular levels of fluorescent BES-So-AM was very big, as estimated by counting the total number of pixels pseudocolored green (in arbitrary units: 50 ± 10 for the wild type, 10 ± 2 for A2, and 153 ± 32 for S2.2; see “Materials and Methods”). The previous result is due to additional BES-So-AM in the cell margins of S2.2 plants.

Next, we used BES-So to detect extracellular $O_2^-$. Similar to the intracellular $O_2^-$, no significant accumulation of BES-So could be detected under control conditions in the three genotypes (Fig. 4B, 0 h). One hour post-treatment, the extracellular BES-So fluorescence increased significantly in S2.2 and the wild type, while it increased moderately in A2 (Fig. 4B, 1 h). Six hours post-treatment, BES-So fluorescence increased further in the wild type and mainly in S2.2, but not in A2 (Fig. 4B, 6 h). Our results indicate that apoplastic PAO levels positively correlate with $O_2^-$ levels in guard cells.

### Apoplastic PAO Levels Correlate with NADPH Oxidase Activity

The correlation between PAO and $O_2^-$ levels in our experiments prompted us to examine the genetic interaction between PAO and two of the major NADPH
oxidase genes in guard cells, RbohD and RbohF (Song et al., 2014). Under control conditions, mRNA levels of RbohD/F were increased significantly in S2.2 compared with the wild type, but not in A2 (Fig. 5A). One and 6 h post-NaCl treatment, the mRNA levels of RbohD tended to increase in all genotypes (Fig. 5A, 1 and 6 h). The same trend, although to a lesser extent, was observed in all genotypes for mRNA levels of RbohF. However, 6 h post-NaCl treatment, the mRNA levels of RbohF decreased slightly in all genotypes compared with 1 h. Under both control and NaCl treatments, the higher mRNA levels of RbohD/F in S2.2 were accompanied by increased in-gel activity of NADPH oxidase, while A2 showed a marked decrease (Fig. 5, B and C, 1 h).

**PAO-Mediated ROS Production Depends on NADPH Oxidase**

Furthermore, we examined the physiological effect of RbohD/F down-regulation in ROS production using plants with silenced RbohD or RbohF (AS-NiRbohD and AS-NiRbohF; Ji and Park, 2011). We observed that RbohF and RbohD mRNA also were reduced in AS-NiRbohD and AS-NiRbohF (Supplemental Fig. S5), respectively. The mRNA of RbohD and RbohF share high sequence similarity (81%; query coverage, 89%), suggesting that the antisense cDNA of RbohD and RbohF can down-regulate RbohF and RbohD, respectively. Therefore, we refer to these transgens hereafter as AS-NiRbohD/F.

Importantly, under control and post-NaCl treatment conditions, the AS-NiRbohD/F plants showed aposlastic NtPAO similar to the wild type (Supplemental Fig. S5). Interestingly, neither O$_2^-$, as expected, nor H$_2$O$_2$ significantly accumulated post-NaCl treatment in the two transgenic genotypes under control and stress conditions (Figs. 6 and 7). These results point to the importance of NADPH oxidase in the production of ROS under short-term NaCl treatment.

In order to confirm the previous result and examine the contribution of PAO/NADPH oxidase to a presumably sustained H$_2$O$_2$ accumulation, we used a pharmacological approach. We used the potent inhibitors diphenyleneiodonium (DPI; 50 $\mu$m) and guazatine (Guaz; 5 $\mu$m) to inhibit NADPH oxidase and PAO,
respectively. Our guard cell assay cannot be used to assay sustained H₂O₂ accumulation, since even the untreated leaf strips die out after approximately 12 h. In order to estimate H₂O₂ for a prolonged time (up to 72 h), we used whole leaves. In all genotypes, DPI ameliorated NaCl-induced H₂O₂ production (Supplemental Fig. S6). These data point out that NADPH oxidase contributes significantly to the accumulation of H₂O₂.

In the presence of Guaz and NaCl, H₂O₂ accumulation was induced relative to the control, albeit to a lesser extent. The strong effect of DPI at early time points (compare 6 h with 72 h) indicates the importance of NADPH oxidase for ROS homeostasis at the onset of stress. As expected, the accumulation of H₂O₂ was further inhibited by the simultaneous addition of both DPI and Guaz, supporting the notion that the two enzymes cooperate in constituting a feed-forward ROS amplification loop.

We should note that DPI inhibits PAO activity among others; however, the potency of this inhibition is much weaker than that of Guaz (Moschou et al., 2008c). We estimated the activity of PAO in the presence of DPI or Guaz. Under our experimental conditions, in the wild type and S2.2, DPI slightly inhibited the apoplastic PAO activity (approximately 15%; Supplemental Fig. S7). However, Guaz nullified the activity of PAO in both genotypes within 6 h. We assume that the weak inhibitory effect of DPI on PAO is not significant.

**DISCUSSION**

In this work, we studied the contribution of the apoplastic PAO and the plasma membrane NADPH oxidase to ROS accumulation and how their cross talk regulates ROS homeostasis. Building on the unexpected observation that PAO regulates O₂⁻ accumulation, the results presented here allow us to propose a model in which a feed-forward amplification loop that involves apoplastic PAO and NADPH oxidase controls ROS accumulation. Our model integrates the observations that apoplastic PAO positively influences the activity of NADPH oxidase and that NADPH oxidase is upstream of PAO in the relay of events that control ROS accumulation. By detailing the relationship between PAO and NADPH oxidase, we could show the absolute requirement of NADPH oxidase for ROS production.
within the first few hours of NaCl treatment. The apoplastic PAO functions as an amplifier of the initial ROS accumulation controlled by NADPH oxidase. Taken together, our model suggests that the apoplastic PAO feeds a stress-inducible ROS amplification loop that can lead to ROS accumulation above a toxicity threshold, culminating in PCD. Our findings allow us to extend our understanding of how apoplastic PAOs control tolerance responses during stresses. Notably, the tissue-wide role of NADPH oxidase and apoplastic PAO in ROS regulation can be detailed in a single-cell context, the guard cells, by the careful selection of specific ROS probes. The observed positive correlation between O$_2$- and apoplastic PAO levels upon short-term NaCl treatment at an organ level (leaf; Fig. 1) could be extrapolated in guard cells (Figs. 2–4). This finding simplifies analyses of ROS accumulation, considering the unique advantages of guard cells as a study system: accessibility for microscopic studies and homogeneity. The latter reason can be quite important considering that different cell types can have different contributions to ROS levels.

But to what extent are the NADPH oxidase and apoplastic PAO important for guard cell physiology? It is well established that both of them contribute to the regulation of stomatal aperture, and this role is...
executed through their intrinsic relation to ROS (Zhang et al., 2009; Fincato et al., 2012, and refs. therein). Loss of RBOHF in Arabidopsis leads to the partial impairment of ABA-induced stomatal closure, which is further reduced, and ROS production is abolished in an AtRbohD/F mutant, suggesting that the two genes act redundantly in the control of stomatal aperture (Chater et al., 2015). In addition, AOs positively contribute to stomatal closure in grapevine (Vitis vinifera; Paschalidis et al., 2010). In contrast, the acetylation of 1,3-diaminopropane, a product of apoplastic PAO by N-ACETYLTRANSFERASE ACTIVITY1 in Arabidopsis, can result in the slowing of stomatal closure (Jammes et al., 2014). Thus, both enzymes are of critical importance to the physiology of stomatal aperture and may act redundantly or cooperatively in the same ROS network.

Feed-forward loops offer an evolutionarily conserved solution to the problem of signal amplification (Cordero and Hogeweg, 2006). Their overabundance in signaling networks most likely reflects their incremental acquisition of adaptive single interactions between different components within the network. Plants have evolved a wide array of feedback loops to control a variety of physiological responses upon various exogenous or endogenous signals. For example, salicylate operates in a feed-forward ROS loop that culminates in cell death (Yun and Chen, 2006). Feed-forward loops for ROS amplification have been described in nonplants as well, between NADPH oxidase and mitochondria-derived ROS (Graham et al., 2012). These loops are subordinate to additional signals, such as metabolic perturbations (e.g. Glc deprivation). Likewise, the PAO/NADPH oxidase loop is subordinate to exogenous stress; activation of this loop requires NaCl treatment. In the absence of NaCl, the loop could not be initiated, even though in S2.2, NADPH oxidase was increased in the controls (Fig. 5). Indeed, under control

Figure 6. Intracellular/extracellular H$_2$O$_2$ in guard cells of wild-type (WT), AS-NtRbohD, and AS-NtRbohF plants post-NaCl treatment. A, CLSM images of intracellular BES-H$_2$O$_2$-Ac fluorescence (green) and chlorophyll (Chl) autofluorescence (red) at 0, 1, and 6 h post-NaCl treatment. White boxes denote nuclei. Images are representative of three independent experiments with six micrographs per genotype in each time point. Bars = 20 μm. B, CLSM images of intercellular AUR fluorescence (red) at 0, 1, and 6 h post-NaCl treatment. Images are representative of three independent experiments with six micrographs per genotype in each time point. Bars = 20 μm.
conditions, the cellular content of $\text{O}_2^{-}$ and $\text{H}_2\text{O}_2$ does not differ significantly among the wild type, A2, and S2.2, as well among the wild type and AS-NtRbohD/F (Figs. 1–4). On the contrary, NaCl treatment dramatically increases both $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$ in S2.2; these ROS increase moderately in the wild type and at very low levels in A2, both intracellularly and extracellularly. Taken together, these findings suggest that the PAO/NADPH oxidase loop is subordinate to yet unidentified signals.

What is the nature of the signals that bring about the activation of the PAO/NADPH oxidase loop? Considering that this loop is activated early after the onset of salinity, it is highly unlikely that it is activated by time-consuming pathways, such as lengthy transcriptional cascade(s). In fact, accumulating evidence supports that NADPH oxidase is amenable to several regulatory posttranslational modifications (Li et al., 2014). Likewise, apoplastic PAO activity may be controlled by posttranslational modifications. In maize, apoplastic PAO activity is controlled by its phosphorylation status (Cona et al., 2006). An alternative scenario would be that the loop is not induced at all but its effect is masked by the ROS-scavenging machinery. In accordance, an adaptive regulation of the ROS-scavenging machinery has been suggested to dispose of surplus $\text{H}_2\text{O}_2$ produced by apoplastic PAO during development (Moschou et al., 2008a). This is supported by the absence of significant ROS accumulation in S2.2, although NADPH oxidase is preinduced in this line (Fig. 5). During stress, a transient decrease of the antioxidant machinery may lead to the unmasking of the effect of the PAO/NADPH oxidase loop that is further enhanced by additional signaling pathways. These two scenarios are not mutually exclusive and may both be plausible, perhaps at different times/phases.

Taking into consideration the potency of the PAO/NADPH oxidase loop to the overall ROS contribution, the next question is to what extent these ROS signal downstream events. A dedicated set of sensor proteins

Figure 7. Intracellular/extracellular $\text{O}_2^{-}$ in guard cells of wild-type (WT), AS-NtRbohD, and AS-NtRbohF plants post-NaCl treatment. A, CLSM images of intracellular BES-So-Am fluorescence (green) and chlorophyll (Chl) autofluorescence (red) at 0, 1, and 6 h post-NaCl treatment. White boxes (black in merged images) denote nuclei. Images are representative of three independent experiments with six micrographs per genotype in each time point. Quantification of green signal is shown at right. Bars = 20 $\mu\text{m}$. B, CLSM images of intercellular BES-So fluorescence (green) and chlorophyll autofluorescence (red) at 0, 1, and 6 h post-NaCl treatment. White boxes denote nuclei. Images are representative of three independent experiments with six micrographs per genotype in each time point.
is involved in the perception of ROS signals (Bosch et al., 2014). These proteins are clustered in networks that mediate signaling events leading to downstream responses, including changes in gene expression and the activation of cell death programs. Our work highlights that the PAO/NADPH oxidase loop has the potential to trigger cell death. Indeed, this loop produces ROS of sufficient quantity to drive protein oxidation and to reach a level of cellular toxicity (Supplemental Fig. S3). Protein oxidation might be the tip of the iceberg in myriad additional cell-wide consequences brought about by the PAO/NADPH oxidase loop, which is set in motion by NaCl treatment and may affect many downstream processes that culminate in cell death execution. Certainly, this loop might just be a hub in a plethora of additional pathways that refine the decision toward cell death. However, it seems likely that the PAO/NADPH oxidase loop possesses a central regulatory role in the execution of cell death, taking into consideration the tight association between apoplastic PAO levels and cell death levels.

An interesting twist to our story is the possible temporal dependence for a PAO/NADPH oxidase loop. The application of DPI significantly affected H2O2 levels mostly at early time points (6 h), while Guaz had a minor effect that was escalated with time (more than 24 h; Supplemental Fig. S6). We speculate that this time-resolved effect of the two inhibitors may indicate the initial importance of the PAO/NADPH oxidase loop; then, PAO is uncoupled from NADPH oxidase and is required to sustain ROS levels. In support of this, AS- NtRbohD/F failed to accumulate O2− and H2O2 (Figs. 6 and 7) during the early stages of salinity, although they contained wild-type-like levels of apoplastic PAO. This finding suggests that NADPH oxidase is upstream of the apoplastic PAO in ROS regulation and that an initial ROS accumulation by NADPH oxidase might be important for triggering the activation of the apoplastic PAO pathway. However, we should note that the interaction between PAO/ NADPH oxidases and their feed-forward relationships do not allow us at this stage to efficiently disentangle their distinct contribution to ROS levels. Considering that inhibitors may be imposed to differential uptake during different stages of stress, our model regarding the temporal emergence of the loop requires further refinement.

Overall, our data suggest that NADPH oxidase and the apoplastic PAO are not parallel pathways for ROS production. Instead, they form a nexus and cross talk in the frame of the strategy of plant cells to regulate ROS homeostasis. In addition, NADPH oxidase and apoplast-ic PAO show a feed-forward relationship in which high PAO levels correlate with high NADPH oxidase activity. Therefore, the two proteins are part of the same ROS homeostatic regulatory module, which affects the extracellular and intracellular cross talk of ROS regulatory mechanisms. However, it is still unclear to what extent intracellular PAOs affect this module. We previously established that, in Arabidopsis, a peroxisomal PAO cross talks with NADPH oxidase to activate the mitochondrial alternative oxidase pathway (Andronis et al., 2014). To advance our understanding of PAO/NADPH oxidase cross talk, the next critical step could be to explore how ROS signals are transduced/perceived for the fine orchestration of this cross talk and the relationship between apoplastic and intracellular PAOs in this regulation.

MATERIALS AND METHODS

Preparation of Transgenic Plants and Growth Conditions

The preparation of transgenic tobacco (Nicotiana tabacum ‘Xanthi’) plants with altered expression of the maize (Zea mays) POLYAMINE OXIDASE (ZmPAO) gene (lines A2 and S2.2) has been described previously (Moschou et al., 2008a, 2008b). The preparation of transgenic tobacco specifically down-regulating the two genes coding NADPH oxidase, RbohD and RbohF, was described by Ji and Park (2011). Surface-sterilized transgenic seeds (3T homozygous) were cultured on solid Murashige and Skoog medium (pH 5.8) and then transferred to soil under light (16/8-h photoperiod and 100 μmol photons m−2 s−1) at 25°C ± 5°C. Two- to 3-week-old-plants were used.

RNA Extraction Quantitative PCR

Total RNA preparation was performed as described previously (Wi and Park, 2002). The primers used (Bionics) are shown in Supplemental Table S1. One microgram of total RNA from leaves was reverse transcribed for 30 min at 42°C in a 20-μL reaction volume using the High Fidelity PrimeScript RT-PCR kit (Takara) according to the manufacturer’s instructions. Quantitative PCR was carried in the Chromo 4 Continuous Fluorescence Detector (Bio-Rad). Cycle threshold values were analyzed using MJ Opticon Monitor Software version 3.1 (Bio-Rad) and then exported to Microsoft Excel for further analysis. The reference gene β-ACTIN was used.

Protein Extraction, Western Blotting, in Gel Enzymatic Assays, and Electrophoresis

Proteins were extracted and treated as described by Papadakis and Rouvelakias-Angelakis (2005). For NADPH oxidase activity staining, the procedure was carried out according to Carter et al. (2007). An aliquot containing 100 μg of protein from each tissue homogenate was electrophoresed on a 10% native PAGE gel. The gel was then incubated in 0.5 mg mL−1 NBT in 10 mM Tris, pH 7.4, supplied with 134 mM NADPH until bands were detected. For PAO activity staining, 50 μg of protein extracts was electrophoretically resolved on a 10% polyacrylamide gel. Subsequently, the gel was incubated in 50 mM phosphate buffer (pH 7) for 30 min, to which 10 mM 5′-adenosine monophosphate, 5′-diphosphate, 5′-triphosphate (AMP-PNP) and 5′-triphosphate (ATP) were added. The gel was rinsed and then incubated in 50 mM phosphate buffer (pH 7) containing 1 mg mL−1 DAB. Protein samples that were incubated with 1 μM Guaz prior to electrophoresis were used as negative controls.

PAO and DAO Enzymatic Assay

The spectrophotometric method developed by Federico et al. (1985) was used to determine apoplastic PAO and DAO activities. Absorbance was read at 460 nm.

Determination of Endogenous PAs

PAs were analyzed as described by Goren et al. (1982). Leaves (0.2 g) were homogenized in 0.5 mL of 5% (v/v) perchloric acid and centrifuged at 15,000 rpm for 20 min. Then, 0.2 mL of saturated sodium carbonate and 0.4 mL of dimethylaminonaphthalene-1-sulfonyl chloride (1 mg mL−1 in acetone) were added to 0.2 mL of the supernatant, and the mixture was incubated at room temperature for 24 h in the dark. The dapsylated products were extracted with benzene and separated by thin-layer chromatography in chloroform:triethylamine (252, v/v). The separated PAs were scraped off and quantified using a spectrofluorimeter (RF-1501; Shimadzu; http://www.shimadzu.com), by which...
the emission at 495 nm was recorded after excitation at 350 nm. Alternatively, PAs were determined as described previously (Kotzabasis et al., 1993) using an HP 1100 high-performance liquid chromatograph (Hewlett-Packard).

**Photometric Determination of H$_2$O$_2$ Levels**

The endogenous levels of H$_2$O$_2$ content of the tissues were determined as described by Sahebani and Hadavi (2009). Fresh leaf material (100 mg) was homogenized in an ice bath with 0.375 mL of 0.1% (w/v) TCA. The homogenate was centrifuged at 7,000 rpm for 20 min, and 0.25 mL of the supernatant was added to 0.25 mL of 10 mM potassium phosphate buffer (pH 7.8) and 0.5 mL of 1 M KI. The absorbance of the supernatant was read at 390 nm. The content of H$_2$O$_2$ was determined using a standard curve.

**In Situ Detection of ROS**

In situ accumulation of H$_2$O$_2$ was detected using the method of Thordal-Christensen et al. (1997) and that of O$_3$$_2$ according to Jabs et al. (1996). In addition, NaCl-treated tobacco leaves were incubated for 2 h in NBT staining solution (1 mg mL$^{-1}$, pH 7.8, 10 mM potassium phosphate buffer) at room temperature. To detect the in situ accumulation of H$_2$O$_2$, NaCl-treated tobacco leaves were incubated for 2 h in DAB staining solution (1 mg mL$^{-1}$, pH 3.8) at room temperature. Tobacco leaves were stained by boiling in 96% (v/v) ethanol and then photographed using a digital camera.

**Confocal Microscopy Detection of ROS in Guard Cells**

For fluorescent detection of ROS, leaf epidermal strips were used. For DCFDA (Sigma Chemical), strips were floated on a solution of 10 mM in 20 mM potassium phosphate buffer (pH 6) for 10 min (excitation, 450 ± 490 nm; barrier, 520 ± 560 nm). AR and AUR (invitrogen) were used at a concentration of 50 mM in 50 mM sodium phosphate buffer (pH 6) for 1 h in the dark (for AR, excitation, 571 nm; emission, 585 nm; for AUR, excitation, 568 nm; emission, 581 nm). BES-H$_2$O$_2$Ac and BES-H$_2$O$_2$ (WAKO Chemicals) were used at a concentration of 20 mM potassium phosphate buffer (pH 6) for 1 h in the dark (excitation, 485 nm; emission, 530 nm). BES-So-A and BES-So (WAKO Chemicals) were used under a concentration of 20 mM potassium phosphate buffer (pH 6) for 1 h in the dark (excitation, 505 nm; emission, 544 nm). Fluorescence was observed using the confocal laser scanning microscope Fluoview 300 (FV 300; Olympus).

**Quantification of DCF in Plant Extracts**

Plant leaves were homogenized with 10 mM Tris buffer (pH 7.2) and then centrifuged at 2,000 g for 5 min. The supernatant was incubated with DCFDA at room temperature for 10 min in the dark. DCF fluorescence was detected by a spectrophotometer (excitation, 485 nm; emission, 525 nm; RF-1501; Shimadzu). Data were expressed as relative fluorescence per milligram of protein.

**Detection of Carbonylated Proteins**

Total proteins from tobacco leaves were extracted from frozen samples by grinding the tissue to a fine powder and resuspended in protein extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail [Sigma Chemical]). The OxyBlot procedure (Millipore) was used to perform immunoblot detection of integrated densitometric values using the OxyBlot procedure. Oxidation index was calculated by the ratio between total proteins and standard protein of pixel-based proteins were detected by an anti-DNP antibody. Oxidation index was calculated by the ratio between total proteins and standard protein of pixel-based proteins.

**Trypan Blue Staining**

To monitor cell death, NaCl-treated tobacco leaf discs were immersed for 1 min in a boiling solution of 10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, and 0.4% (w/v) Trypan Blue. After leaf discs had cooled down to room temperature, the solution was replaced with 70% (w/v) chloral hydrate. Leaf discs were destained overnight and then photographed using a digital camera.

**Statistical and Image Analyses**

Statistical analysis was carried out with SigmaPlot 12.0 statistical software. After ANOVA, Duncan’s multiple comparisons were performed. Image analysis was done using Fiji software (Schindelin et al., 2012). For image quantifications of NBT and DAB, we selected 10 regions of interest (five on each leaf side) of the same area (rectangular) and quantified the integrated density in inverted color images. These 10 measurements corresponded to a technical replicate. For quantification of fluorescent signals, the same approach was used. For the total green pixel count, we used the Adjust > Color Threshold in Fiji and regions of interest that included the guard cells. For Fiji analyses, the methods described by Moschou et al. (2013, 2016) were used.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *Zea mays POLYAMINE OXIDASE*, NC_024468; *Nicotiana tabacum POLYAMINE OXIDASE*, NP_00313231; *Nicotiana tabacum RESPIRATORY BURST OXIDASE HOMOLOG F*, EU072744; and *Nicotiana tabacum RESPIRATORY BURST OXIDASE HOMOLOG D*, EF366670.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Endogenous PA levels in the leaves of wild-type, A2, and S2.2 transgenic plants under control and 24 h post-NaCl treatment.

**Supplemental Figure S2.** PA catalytic genes/enzymes in wild-type, A2, and S2.2 transgenic plants under control and post-NaCl treatment.

**Supplemental Figure S3.** PCD hallmarks in wild-type, A2, and S2.2 leaves post-NaCl treatment.

**Supplemental Figure S4.** Intracellular/extracellular H$_2$O$_2$ in guard cells of wild-type, A2, and S2.2 plants post-NaCl treatment.

**Supplemental Figure S5.** Relative mRNA levels of *PAO, RbohD*, and *RbohF* genes in *AS-NrRbohD* and *AS-NrRbohF* plants post-NaCl treatment.

**Supplemental Figure S6.** H$_2$O$_2$ levels in leaves 6, 24, 48, and 72 h post-NaCl treatment in the absence or presence of DPI, Guaz, or both.

**Supplemental Figure S7.** Apoplastic PAO activity in the presence of DPI post-NaCl treatment.

**Supplemental File S1.** PAO and DAO genes in tobacco.

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**LITERATURE CITED**


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