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Review

Research review

Production, amplification and systemic propagation of redox messengers in plants? The phloem can do it all!

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Summary

Rapid long-distance signalling is an emerging topic in plant research, and is particularly associated with responses to biotic and abiotic stress. Systemic acquired resistance (SAR) to pathogen attack is dependent on nitric oxide (NO) and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). By comparison, systemic wound responses (SWRs) and systemic acquired acclimation (SAA) to abiotic stress encounters are triggered by rapid waves of H_2O_2, calcium and electrical signalling. Efforts have been made to decipher the relationship between redox messengers, calcium and other known systemic defence signals. Less is known about possible routes of signal transduction throughout the entire plant. Previously, the phloem has been suggested to be a transport conduit for mobile signals inducing SAR, SWR and SAA. This review highlights the role of the phloem in systemic redox signalling by NO and ROS. A not yet identified calcium-dependent NO source and S-nitrosoglutathione reductase are candidate regulators of NO homeostasis in the phloem, whereas ROS concentrations are controlled by NADPH oxidases and the H_2O_2-scavenging enzyme ascorbate peroxidase. Possible amplification mechanisms in phloem-mediated systemic redox signalling are discussed.

Introduction

The plant vascular system consists of phloem, xylem and parenchyma cells (Lucas *et al.*, 2013). While xylem vessels supply plant organs with water and minerals taken up by the roots, the phloem distributes photoassimilates from photosynthetically active source leaves to sinks such as roots, young leaves, fruits and meristems. Sieve tubes are the transport conduits of the phloem, consisting of elongated cells, called sieve elements (SEs), which are connected by perforated sieve plates. The enucleate SEs are supplied with vital compounds by the companion cells (CCs). As a consequence of their high nutrient content, vascular bundles are attractive targets for insects and pathogens. For instance, aphids, whiteflies and leafhoppers are specialized in phloem feeding (Douglas, 2006) whereas viruses and small cell-wall-free Mollicutes bacteria, such as mycoplasmas and phytoplasmas, propagate inside the sieve tubes, utilizing them as systemic highways for the infection of distal plant parts (Buxa *et al.*, 2015). Yet, the xylem also is colonized by vascular wilt pathogens such as the ascomycete fungus *Fusarium oxysporum* f. sp. *lycopersici* and the bacterium *Ralstonia solanacearum* (Yadeta & J Thomma, 2013). Diseases caused by vascular pathogens are difficult to control, suggesting that elucidating the defence mechanisms against such diseases would help to prevent severe agronomic yield losses.

Phloem and xylem do not only respond to local cues but also act as a route for long-distance signalling (Dempsey & Klessig, 2012; Gaupels & Corina Vlot, 2012; Lucas *et al.*, 2013). For instance, phloem transport of the protein FLOWERING LOCUS T is involved in floral induction, while mobile mRNAs corresponding to the genes *GIBBERRELIC ACID INSENSITIVE* and *KNOTTED-like* regulate leaf development (Lucas *et al.*, 2013). Systemic signalling is a common phenomenon in plant stress responses, the most extensively studied being systemic acquired resistance (SAR) upon local pathogen infection. This broad-spectrum, long-lasting type of enhanced immunity is dependent on salicylic acid (SA) in remote but not in locally infected tissues (Dempsey & Klessig, 2012). By contrast, leaf damage by insect feeding triggers a systemic wound response (SWR) upon local

perception of herbivore- or damage-associated elicitors (Erb *et al.*, 2012). Both local and systemic wound responses are dependent on jasmonic acid (JA) (Erb *et al.*, 2012). Finally, averse environmental conditions, such as excess light and heat, trigger systemic acquired acclimation (SAA) responses involving the phytohormone abscisic acid (ABA) (Karpinski *et al.*, 1999; Galvez-Valdivieso *et al.*, 2009).

Disruption of phloem transport by stem girdling provided evidence that systemic defence responses are dependent on phloem-bound signalling (Gaupels & Corina Vlot, 2012). Notably, JA and SA were found to be synthesized in phloem and parenchyma cells serving both in phloem-internal and long-distance signalling (Dempsey & Klessig, 2012; Gaupels & Corina Vlot, 2012). The role of the xylem in defence responses is largely unknown, although SA, JA and ABA were all shown to be present in both xylem and phloem exudates (Furch *et al.*, 2014). Recent research uncovered calcium-dependent electrical signals, hydrogen peroxide (H_2O_2), and nitric oxide (NO) as new players in systemic stress responses (Wendehenne *et al.*, 2014; Gilroy *et al.*, 2016). Experiments with the plant model *Arabidopsis thaliana* demonstrated that NO and reactive oxygen species (ROS) are required for the establishment of SAR, while a calcium-ROS auto-propagation wave interacts with electric signals for induction of SWR and SAA. Leaf wounding also triggered NO and ROS production in the vascular tissues of various plant species (Corpas *et al.*, 2008; Gaupels *et al.*, 2016).

The present review is centred upon systemic stress signalling by NO and ROS. We will summarize evidence for synthesis and mobility of these messengers within the vascular bundles, discuss possible interactions with known systemic defence signals, and assess how NO and ROS, as rather unstable molecules, can be propagated over long distances through amplification loops.

NO and ROS are produced within the vascular bundles

NO and ROS are general stress messengers that accumulate upon pathogen attack and various abiotic stresses such as wounding and heat (Besson-Bard *et al.*, 2008; Mignolet-Spruyt *et al.*, 2016). After inoculation of plants by avirulent pathogens, NO and H_2O_2 act synergistically to induce the hypersensitive response (HR) which culminates in programmed cell death (Besson-Bard *et al.*, 2008). Cross-talk between NO and ROS is also required for the pathogen- and ozone-induced regulation of gene expression (Zago *et al.*, 2006; Ahlfors *et al.*, 2009). The NADPH oxidases RESPIRATORY BURST OXIDASE HOMOLOGUE D and F (RBOHD/F) and peroxidases are major sources of ROS in plants (Kadota *et al.*, 2015; Mignolet-Spruyt *et al.*, 2016). RBOHD produces superoxide that is efficiently converted to H_2O_2 by superoxide dismutase. NO is mainly produced by a yet unknown NO synthase (NOS)-like enzyme (Besson-Bard *et al.*, 2008). Additionally, NO can derive from nitrite either nonenzymatically at low pH or via nitrate reductase (NR) activity (Besson-Bard *et al.*, 2008). Within the cytoplasm, NO efficiently binds to glutathione through cysteine S-nitrosylation, thereby forming S-nitrosoglutathione (GSNO). Cellular concentrations of NO and GSNO are controlled by the enzyme GSNO reductase (GSNOR) which decomposes GSNO to oxidized glutathione and ammonium (Yu *et al.*, 2014). In contrast, H_2O_2 concentrations are regulated by antioxidant enzymes including ascorbate peroxidases (APXs) and catalases (CATs) (Romero-Puertas & Sandalio, 2016).

Using microscopic approaches with specific fluorescent dyes, NO and ROS have been detected in vascular bundles of different plant species under a number of stress conditions (Valderrama *et al.*, 2007; Corpas *et al.*, 2008; Tanou *et al.*, 2009). Often fluorescence was more prominent in vascular bundles compared to other tissues. For instance, salt stress in roots of citrus and olive (*Olea europaea*) trees triggered NO and ROS synthesis mainly in leaf veins (Valderrama *et al.*, 2007; Tanou *et al.*, 2009), although the specific vascular cell types and enzymatic activities have not been further defined. We investigated defence signalling in the living vascular tissues of *Vicia faba* (Gaupels *et al.*, 2008). Shallow cortical cuts into the leaf mid veins created a window for microscopic observation and treatment of the exposed vascular strands. Adding the fungal elicitor chitooctaose and defence signals such as SA and H_2O_2 induced strong fluorescence of the NO-specific dye diaminofluoresceine in the phloem (Fig. 1) (Gaupels *et al.*, 2008). The NO burst was dependent on calcium and could be blocked by inhibitors of NOS, but not by NR inhibitors or by inhibitors of the mitochondrial electron transport chain. Significantly, NO was detected in SEs, suggesting its systemic transport to sink tissues (Fig. 1c,d).

Fabaceae are the only group of plants to have forisome proteins, which function in sieve tube occlusion by rapid calcium-dependent dispersion (Van Bel *et al.*, 2014). In *V. faba*, this forisome response was triggered after treating the phloem with H_2O_2, suggesting a calcium influx into the SEs (Fig. 1c). Within the sieve tubes, calcium could even serve as a long-distance messenger (Van Bel *et al.*, 2014). It is important that H_2O_2 induced rapid NO synthesis mainly in CCs and vascular parenchyma cells but not, or to a much lesser extent, in other tissues (Figs 1, 2). Hence, the phloem seems to be particularly sensitive to H_2O_2 and is well equipped with NO-generating enzymes (Gaupels *et al.*, 2008). Collectively, the described findings suggest that vascular tissues are a site of signal interactions between NO, ROS and calcium.

SAR involves NO and ROS signalling in the phloem

The nature of the mobile SAR inducer is still unclear (Dempsey & Klessig, 2012). Candidate signals include methyl salicylate (MeSA), azelaic acid (AzA), glycerol-3-phosphate (G3P), dehydroabietenal (DA) and pipecolic acid (Dempsey & Klessig, 2012; Lucas *et al.*, 2013). Mounting evidence also suggests the involvement of NO signalling in SAR (Gaupels, 2015). Injection of NO donors into tobacco (*Nicotiana tabacum*) leaves reduced the size of lesions caused by tobacco mosaic virus (TMV) on treated and systemic nontreated leaves, whereas NOS inhibitors and an NO scavenger attenuated SAR in distal leaves (Song & Goodman, 2001). Rusterucci *et al.* (2007) proposed GSNO as a systemic signal based on the observation that *A. thaliana GSNOR1* antisense (*GSNOR1-AS*) lines displayed elevated GSNO concentrations and constitutive SAR against *Hyaloperonospora parasitica*. Moreover, GSNOR is primarily located in CCs, suggesting that inhibition of the enzyme or down-regulation of its gene expression promotes the

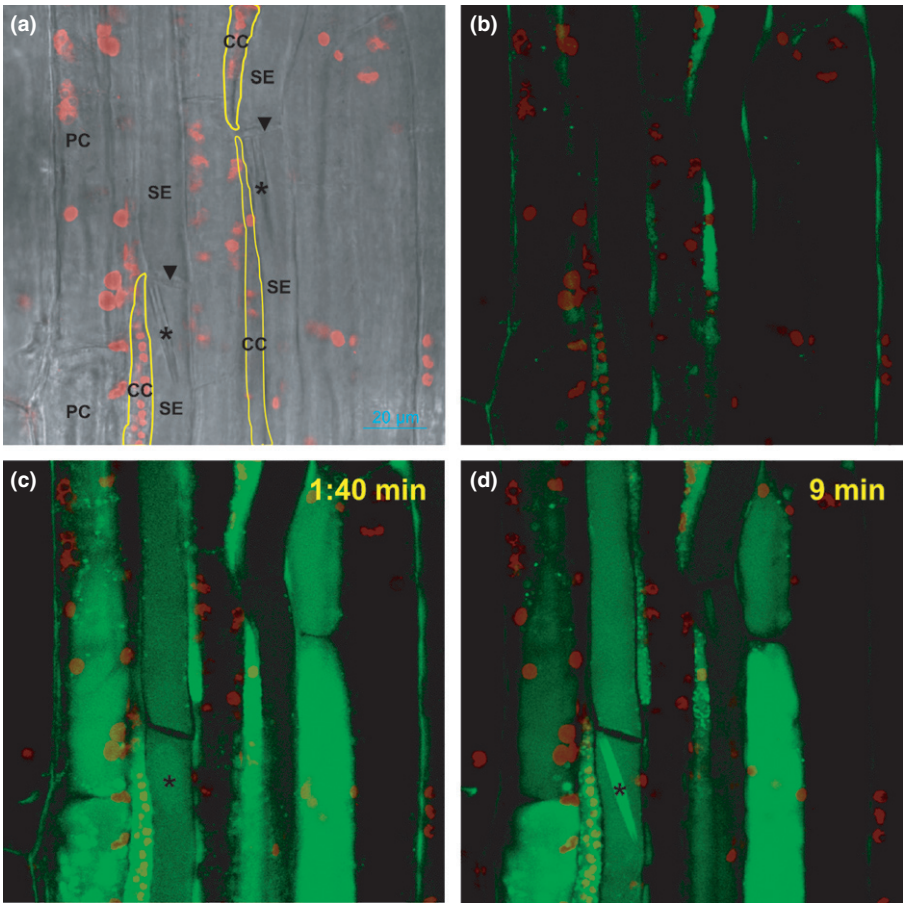

Fig. 1 Hydrogen peroxide (H_2O_2)-induced nitric oxide (NO) production in the phloem of *Vicia faba*. NO was detected by confocal laser scanning microscopy using the fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2DA). (a) Digital overlay of light transmission image and UV-induced chloroplast autofluorescence (red). Condensed calcium-sensitive forisomes (asterisks) and sieve plates (arrow heads) between sieve elements (SE) are indicated. Companion cell borders (CCs) are highlighted by yellow lines. Two elongated vascular parenchyma cells (PCs) are depicted. (b–d) Overlay of DAF fluorescence (green) and chloroplast autofluorescence (red) before (b) and after treatment with 1 mM H_2O_2. Forisomes dispersed at 1 min 40 s (c) but re-condensed at 9 min after H_2O_2 treatment (d), indicative of a transient calcium influx into the SEs. The figure is modified after Gaupels *et al.* (2008).

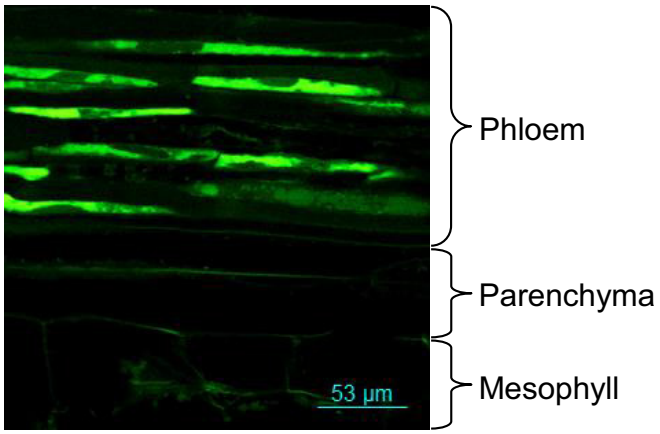

Fig. 2 Hydrogen peroxide (H_2O_2)-induced nitric oxide (NO) production in phloem companion cells of *Vicia faba*. Companion cells can be easily recognized by their spindle-like shape. Note that neither the elongated vascular parenchyma cells nor mesophyll cells were stained by 4,5-diaminofluorescein diacetate (DAF-2DA) after treatment with 10 mM H_2O_2. The figure is modified after Gaupels *et al.* (2008).

accumulation and transport of GSNO in the sieve tubes, which may be an important factor in the induction of SAR (Rusterucci *et al.*, 2007). In systemic leaves, GSNO could induce expression of defence genes similar to its local effect in tobacco leaves (Durner *et al.*, 1998; Yu *et al.*, 2014).

Contrary to the above findings, other researchers reported that *A. thaliana* T-DNA insertion mutants of *GSNOR1* (*gsnor1*) were more susceptible to pathogens than wild-type plants, arguing for a role of NO and GSNO as negative regulators of pathogen resistance (Feechan *et al.*, 2005). The issue has been finally settled by a careful investigation of SAR in *A. thaliana* plants treated with NO and ROS donors and mutants with altered NO and ROS concentrations (Wang *et al.*, 2014). Experiments with the NO donors diethylenetriamine dinitric oxide (DETA-NONOate) and sodium nitroprusside demonstrated that NO induced systemic immunity against the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 in a dose-dependent manner. Notably, injection of 100 µM DETA-NONOate triggered much stronger resistance in distal leaves than 300 µM. Consistent with this, SAR was suppressed in *gsnor1* mutants that accumulate high concentrations of NO. Accordingly, intermediate NO concentrations in *GSNOR1-AS* plants, showing only partially reduced GSNOR activity, would rather stimulate SAR, analogous to intermediate NO donor concentrations (Wang *et al.*, 2014).

ROS donors stimulated systemic immunity in a similar fashion to NO donors (Alvarez *et al.*, 1998; Wang *et al.*, 2014). Moreover, ROS-induced SAR was compromised in NO-deficient mutants, while, in contrast, NO-induced SAR was compromised in *rbohD* and *rbohF* mutant plants, suggesting that the two redox signals cooperate in a positive feedback loop (Wang *et al.*, 2014). SAR induction by both H_2O_2 injection and infection with avirulent *Pst*

was suppressed by the NADPH oxidase inhibitor diphenyliodonium and by H_2O_2-degrading CAT (Alvarez *et al.*, 1998). Apart from RBOHD and RBOHF, an extracellular peroxidase has been identified as an alternative source of H_2O_2 in local and systemic ROS signalling during SAR in pepper (*Capsicum annuum*) plants (Choi *et al.*, 2007). Local and systemic resistance of *A. thaliana* against *Pst* DC3000 involves CALCIUM-DEPENDENT PROTEIN KINASE5 (CPK5) (Dubiella *et al.*, 2013). RBOHD was demonstrated to be phosphorylated and thereby activated by CPK5 *in vivo*, while CPK5 in turn was activated by H_2O_2, suggesting that a pathogen-triggered calcium-CPK5-RBOHD circuit is essential for the onset of SAR (Dubiella *et al.*, 2013).

Taken together, the available data support a role of redox signalling by NO and ROS in systemic immunity to microbial pathogens. SAR is at least to a large extent mediated by the phloem (Gaupels & Corina Vlot, 2012; Lucas *et al.*, 2013), and GSNOR is an important modulator of SAR. However, whether GSNO and H_2O_2 are mobile in the phloem remains to be shown. Moreover, the interactions between NO, ROS, calcium and other known SAR signals are also not well understood (Wendehenne *et al.*, 2014).

ROS, calcium and NO signalling in the phloem during SWR and SAA

ROS and NO have also been implicated in systemic responses to a number of biotic and abiotic stresses, indicating that they are general stress messengers (Gilroy *et al.*, 2016; Mignolet-Spruyt *et al.*, 2016). Wounding, excess light, heat, and salt caused a rapidly spreading wave of ROS production, as demonstrated in transgenic *A. thaliana* plants expressing the luciferase (LUC) reporter gene under control of the ROS-inducible *ZINK FINGER OF ARABIDOPSIS THALIANA 12* promoter (Miller *et al.*, 2009; Suzuki *et al.*, 2013). The ROS wave was interrupted by pretreatment of stem sections with CAT or a calcium channel blocker, pointing to functions of H_2O_2 and calcium in the systemic signal propagation (Miller *et al.*, 2009; Gilroy *et al.*, 2016).

Local leaf damage also triggered the systemic propagation of a calcium-driven electric signal, which moved along the phloem independently of the assimilate flow (Rhodes *et al.*, 1996; Salvador-Recatalà *et al.*, 2014). Intracellular calcium transients were directly or indirectly controlled by clade 3 GLUTAMATE RECEPTOR-LIKE (GLR) channels and TWO PORE CHANNEL1 (TPC1) (Mousavi *et al.*, 2013; Gilroy *et al.*, 2016; Hedrich *et al.*, 2016). Both H_2O_2 signalling and electrical signalling were suppressed in *rbohD* plants (Miller *et al.*, 2009; Suzuki *et al.*, 2013), and further experimental approaches combined with mathematical modelling confirmed that rapid systemic signalling is mediated by a H_2O_2-calcium autopropagation wave (Fig. 3a) (Evans *et al.*, 2016). Local leaf squeezing causes pressure changes in the phloem that can be transmitted over long distances. Such hydraulic waves might be linked to JA and electrical signalling in the phloem via mechanoreceptor-induced calcium fluxes (Farmer *et al.*, 2014). Calcium would also connect hydraulic signals with the NADPH oxidase-driven ROS wave.

GSNO was hypothesized to act as a phloem-mobile carrier of NO during SWR in *A. thaliana* (Espunya *et al.*, 2012). After leaf wounding, accumulation of GSNO in the systemic leaf started in the main vein and subsequently spread throughout the leaf blade. Whether GSNO moved over long distances or arose locally within the phloem is not known. As mentioned before, GSNOR is mainly localized in CCs, which makes this enzyme an excellent candidate modulator of stress signalling by NO/GSNO. NO binds to proteins by S-nitrosylation of cysteine residues, whereas peroxynitrite, which is the reaction product of NO and superoxide, modifies proteins by nitration (NO_2 adduct) of tyrosine or tryptophane residues (Besson-Bard *et al.*, 2008; Yu *et al.*, 2014). As NO is produced in the phloem, one would expect phloem proteins to be modified by S-nitrosylation and/or tyrosine nitration under stress conditions. Indeed, Valderrama *et al.* (2007) visualized nitrated and S-nitrosylated proteins in the vascular tissue of salt-stressed olive plants using antibodies and fluorescence probes in a microscopic analysis. However, no attempt was undertaken to biochemically identify the NO-modified proteins.

Phloem sap can be easily sampled from cut petioles and stems of pumpkin (*Cucurbita maxima*) plants. The exuding droplets derive from the extrafascicular phloem (EFP), which is specialized in defence against herbivorous insects (Gaupels & Ghirardo, 2013).

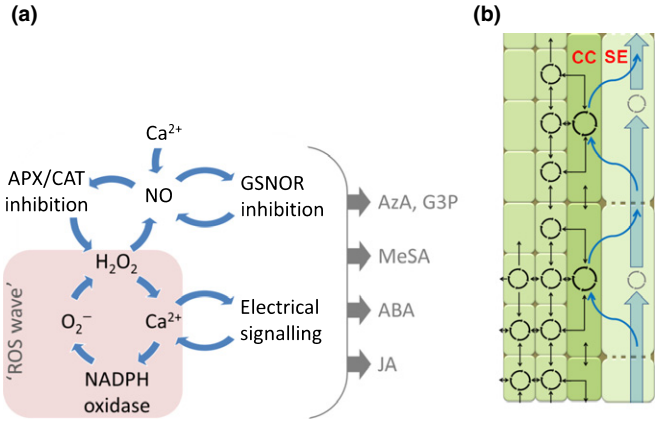

Fig. 3 Local amplification and systemic propagation of defence signals in the phloem. (a) The model depicts hypothetical interactions between known systemic signals. Hydrogen peroxide (H_2O_2) induces nitric oxide (NO) production while NO in turn facilitates H_2O_2 signalling by inhibition of the antioxidant enzymes ascorbate peroxidase (APX) and catalase (CAT). NO inhibits S-nitrosoglutathione reductase (GSNOR) which in turn facilitates further accumulation of NO. Calcium (Ca^{2+}), the Ca^{2+}-dependent synthesis of superoxide (O_2^-) by NADPH oxidases, and H_2O_2 constitute the 'reactive oxygen species (ROS) wave' (pink box). Ca^{2+} could provide a link between ROS production by NADPH oxidases, NO production by a not yet identified enzyme, and electrical signalling. NO and ROS are involved in methyl salicylic acid (MeSA)-, azelaic acid (AzA)-, glycerol-3-phosphate (G3P or derivatives thereof)- and abscisic acid (ABA)-mediated responses, while electric signals trigger jasmonic acid (JA) production. (b) Signal amplification loops propagate most rapidly in the companion cell (CC)/sieve element (SE) complexes of the phloem which is optimized for transport processes. The metabolically highly active CCs are probably a major site of signal synthesis. Some signals such as JA are even produced within the SEs. Blue arrows indicate phloem-internal signalling. Within the phloem, signal propagation waves could move bidirectionally with, as well as against, the assimilate flow. Systemic signal transmission in tissues other than the sieve tubes is possible but less efficient (black arrows). The xylem is not shown because of a lack of data on signal transmission in this tissue. Vascular parenchyma is not shown for the sake of clarity. The figure is modified after Gaupels (2015).

Western blot analyses with antibodies against nitrotyrosine revealed the accumulation of nitrated proteins in phloem exudates of pumpkin plants upon watering with 10 mM H_2O_2 (Gaupels *et al.*, 2008). Local leaf squeezing triggered SWR in the EFP, including JA signalling and subsequent changes in the composition of phloem proteins and metabolites (Gaupels *et al.*, 2012). S-nitrosylation of phloem proteins – as visualized by the biotin switch method – was transiently increased at 1 h but decreased at later time-points, whereas tyrosine nitration showed a continuous increase from 1 to 48 h after wounding (Gaupels *et al.*, 2016). The 16-kD PHLOEM PROTEIN-1 (PP16-1), CYCLOPHILIN 18 (CYP18), and PHLOEM PROTEIN-2 (PP2) were modified by oxidation, S-nitrosylation and tyrosine nitration and might represent central redox sensors within the phloem (Gaupels *et al.*, 2012, 2016).

In sum, SWR and SAA rely on systemic signalling by calcium-dependent electric signals, a H_2O_2-calcium autopropagation wave and NO. Calcium connects electric signals and H_2O_2 (Fig. 3a). As mentioned previously, calcium was also shown to be essential for H_2O_2-induced NO production in the phloem (Gaupels *et al.*, 2008). Collectively, these findings indicate that second messengers cooperate with phytohormones in plant stress responses. However, the exact modes of interactions in systemic signalling events remain to be deciphered.

Specificity of systemic redox and calcium signalling

Rapid systemic signalling exhibited a certain degree of stimulus specificity. Wounding, excess light, heat and salt triggered the rapid ROS production wave but heat exposure additionally induced ABA, while wounding mainly induced JA along with ABA, as inferred from transcriptomic data and phytohormone measurements (Fig. 3a) (Miller *et al.*, 2009; Suzuki *et al.*, 2013). Experiments with *A. thaliana* lines expressing the LUC reporter gene driven by the *ASCORBATE PEROXIDASE2* promoter revealed that cooperative action of ABA and H_2O_2 in vascular cells is essential for the induction of SAA in response to excess light stress (Karpinski *et al.*, 1999; Galvez-Valdivieso *et al.*, 2009). In relation to previously identified SAR signals, NO and ROS were proposed to be upstream of AzA and G3P but independent of SA and MeSA (Wang *et al.*, 2014). The latter notion needs clarification by future studies because other researchers reported cooperative signalling by NO, ROS and SA in resistance (Durner *et al.*, 1998; Feechan *et al.*, 2005; Rusterucci *et al.*, 2007; Gaupels *et al.*, 2008; Espunya *et al.*, 2012).

Interactions between calcium and phytohormones were corroborated by the observation that CPK5-promoted local and systemic pathogen resistance was accompanied by the accumulation of SA (Fig. 3a) (Dubiella *et al.*, 2013). The different systemic signalling events discussed here have still not been fully elucidated, but it is noteworthy that they are all dependent on NADPH oxidases and calcium (Gilroy *et al.*, 2016; Hedrich *et al.*, 2016). Therefore, the question arises of how rather simple molecules such as calcium, ROS and NO can transmit stimulus-specific messages over long distances. Mechanisms of specificity could be related to the strength, duration and signature of signalling (Dodd *et al.*, 2010; Cui *et al.*, 2015). For instance, infection of plants with avirulent pathogens triggers biphasic waves of calcium, ROS and NO. The small early peak and the prolonged second wave of signals were shown to induce qualitatively different defence responses (Cui *et al.*, 2015). Particularly, ROS- and NO-regulated mitogen-activated protein kinases as well as calcium-dependent protein kinases are candidate control units of response specificity (Dodd *et al.*, 2010; Cui *et al.*, 2015).

Alternatively, multi-layered responses are initiated after stress perception by the plant (Gaupels, 2015). Rapid general stress signalling involves ROS, calcium, NO and electric signals, which together might regulate the shift from primary to secondary metabolism. Phytohormones would provide distal plant parts with additional stress-specific information (Fig. 3a). In any case, the exact mechanisms of specificity in systemic signalling events remain unclear.

Systemic propagation of the unstable redox messengers ROS and NO by amplification loops

During local stress responses, NO facilitates the accumulation of ROS by inhibition of antioxidant enzymes. Particularly, the H_2O_2-scavenging enzymes ascorbate peroxidase (APX) and catalase are often down-regulated under severe stress conditions (Fig. 3a) (Romero-Puertas & Sandalio, 2016). In heat-exposed tobacco suspension cells, APX was inhibited by NO-mediated S-nitrosylation, thereby causing an increase in H_2O_2 concentrations. NO synthesis in turn was induced by H_2O_2, placing both signals in a positive feedback loop (de Pinto *et al.*, 2013). Accordingly, SAA in response to heat shock was improved in the *apx1* mutant compared with wild-type plants, suggesting a role of APX in the control of systemic signalling by H_2O_2 (Suzuki *et al.*, 2013). In line with this assumption, local leaf wounding caused a systemic down-regulation of APX activity in the pumpkin EFP (Gaupels *et al.*, 2016). The decrease in APX activity correlated well with an increase in protein S-nitrosylation and tyrosine nitration during the SWR. Future work will reveal whether the APX activity is inhibited by NO modifications. The wound-induced inhibition of APX and the observed reduction in total antioxidants within the sieve tubes would facilitate the systemic transport of H_2O_2 in the phloem.

Another point of intersection between ROS and NO signalling is calcium. RBOHD is activated by calcium and calcium-dependent protein kinases (Kadota *et al.*, 2015), while ROS trigger calcium transients during stress responses (Mignolet-Spruyt *et al.*, 2016). These signal interactions drive the H_2O_2 autopropagation wave (Gilroy *et al.*, 2016). Calcium influx into the SEs is essential for sieve tube occlusion by callose formation and dispersion of forisomes in *V. faba* (Van Bel *et al.*, 2014). Application of H_2O_2 to the phloem induced calcium-dependent forisome dispersion and a rapid calcium-dependent NO burst (Gaupels *et al.*, 2008). Considering this, it seems feasible that calcium is an important mediator of ROS–NO cooperation within the phloem during the systemic propagation of a local alarm status.

Amplification loops are currently emerging as a widespread phenomenon in systemic defence signalling (Wendehenne *et al.*,

2014; Gilroy *et al.*, 2016). Particularly, the reactive molecules H_2O_2 and NO would get lost during long-distance transport as a result of dilution and scavenging. For this reason, they must be constantly synthesized *en route* (Gaupels, 2015). In addition to synthesis, the removal of signals from the cell must also be tightly controlled. This is necessary for shaping the amplitude, speed and duration of signalling but also for scavenging the redox-active molecules before they reach toxic concentrations. For instance, in CCs – as in other cell types – GSNOR maintains low basal concentrations of GSNO and NO. It was recently shown that high concentrations of NO inhibited GSNOR by S-nitrosylation (Frungillo *et al.*, 2014). Hence, under stress conditions, NO could promote its own systemic translocation by inhibiting GSNOR in CCs (Fig. 3a).

NO is also directly involved in the regulation of RBOHD. Inhibition of this enzyme by S-nitrosylation is thought to be a mechanism for preventing excess cell damage and death in *A. thaliana* (Yun *et al.*, 2011). In such a scenario, intermediate NO concentrations would enhance ROS accumulation by inhibition of antioxidant enzymes (Fig. 3a), whereas high NO concentrations at later stages of the stress response would blunt RBOHD activity in order to avoid uncontrolled ROS-calcium-RBOHD amplification. Moreover, H_2O_2 influences its own stability in the vasculature by (indirect) stimulation of the *APX2* gene, which is expressed specifically in bundle sheet parenchyma cells (Karpinski *et al.*, 1999; Galvez-Valdivieso *et al.*, 2009). It was proposed that H_2O_2 acts as a systemic signal moving in the phloem while APX2 activity in the bundle sheet parenchyma modulates H_2O_2 concentrations and confines signalling to the vasculature (Karpinski *et al.*, 1999). Such examples illustrate the complex regulatory mechanisms required for systemic redox signalling.

In general, signal propagation waves can move from cell to cell in all tissues but are most efficiently transmitted in the vasculature. In the phloem, CCs and SEs are tightly interconnected by special pore-plasmodesma units (PPUs) (Lucas *et al.*, 2013). Sieve tubes consist of a string of SEs, which are separated only by the largely perforated sieve plates, while CCs are connected to each other by numerous PPUs. As a consequence, phloem strands constitute a symplastic entity for efficient low-resistance transport of assimilates and stress messengers (Fig. 3b) (Gaupels, 2015). Signal propagation waves in the phloem can also move against the assimilate flow, as demonstrated for the calcium/electric wave which displayed an apparent transmission velocity of 0.3 mm s^{-1} (Salvador-Recatalà *et al.*, 2014). Notably, the ROS wave moves acro- and basipetally with a similar velocity of 0.14 mm s^{-1} (Miller *et al.*, 2009) although transduction in the phloem remains to be investigated.

In sum, the systemically moving ROS−calcium loop probably induces concomitant NO production, which further drives ROS accumulation by inhibition of antioxidant enzymes along the signalling route. Collective evidence strongly suggests that these systemic signals move in the phloem.

Conclusions and future perspectives

H_2O_2 and GSNO can move from the initial site of stress encounter to distal plant parts, thereby participating in the induction of systemic stress immunity to pathogens and pests as well as tolerance to abiotic encounters. In this context, the finding is important that the phloem itself can synthesize these redox signals and might be the transport route for a rapid ROS-calcium-NO autopropagation wave. NO thereby facilitates ROS accumulation by inhibiting antioxidant enzymes, as it has been observed during local defence responses. H_2O_2, in turn, was shown to be a potent inducer of calcium-dependent NO production in the phloem. The described signalling events probably occur during both phloem-internal and systemic stress adaptations. Perhaps the intensity of the initial stimulus determines whether local amplification turns into systemic propagation of redox signalling. Similar apparent translocation velocities also suggest a link between rapid redox and electrical signalling. At least calcium-driven electric signals were shown to move along the phloem.

Collectively, the summarized research implies that we must say goodbye to the old idea that a single specific messenger induces SAR, SWR or SAA. Communication between distal plant parts rather involves sequential and parallel signalling events. For instance, systemic wound responses are regulated by electrical signals, ROS, NO and JA. By flexibly combining partly independent signalling pathways, the plant might optimize both the speed and specificity of the systemic defence response. Future research will have the challenging task of defining the molecular basis of signal interactions within the phloem. To this end, the most promising experimental approaches include real-time imaging of the living phloem as well as direct biochemical analyses of phloem exudates.

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