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Plant innate immunity – sunny side up?

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Abstract

Reactive oxygen species (ROS)- and calcium- dependent signaling pathways play well-established roles during plant innate immunity. Chloroplasts host major biosynthetic pathways and have central roles in energy production, redox homeostasis, and retrograde signaling. However, the organelle's importance in immunity has been somehow overlooked. Recent findings suggest that the chloroplast also has an unanticipated function as a hub for ROS- and calcium-signaling that affects immunity responses at an early stage after pathogen attack. In this opinion article, we discuss a chloroplastic calcium-ROS signaling branch of plant innate immunity. We propose that this chloroplastic branch acts as a light-dependent rheostat that, through the production of ROS, influences the severity of the immune response.

Keywords

chloroplast; calcium signaling; reactive oxygen species signaling; pathogen-triggered immunity; effector-triggered immunity; light; pathogen

ROS and calcium form the basic ingredients for plant innate immunity

Immunity is essential for plants to cope with pathogen infections, which would otherwise lead to dramatic losses in agriculture currently prevented through intensive and costly pest management strategies [1,2]. Upon infection, plant pathogenic fungi, oomycetes, viruses,

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and bacteria face the plant cell wall as a first barrier. When this barrier is breached, the pathogens are recognized by plant membrane pattern recognition receptors (PRRs) (see Glossary). These receptors can recognize microbial molecules, such as protein or cell wall derivatives that are often conserved in the same class of microbes, thereby activating pathogen- or microbial-associated molecular pattern (PAMP; MAMP)-triggered immunity (PTI; MTI) [3]. In order to inhibit PTI, pathogens can secrete virulence effector proteins into the plant cells, which in turn can be recognized by intracellular plant receptors of the nucleotide-binding and leucine-rich repeat domain class (NB-LRRs), thereby activating effector-triggered immunity (ETI). This second layer of immunity often culminates with a hypersensitive response (HR) programmed cell death [4]. Failure by the plant to recognize PAMPs or effector proteins paves the way for successful pathogen infection.

Over the past 3 decades, ROS have been well established as an integral aspect of plant immunity in a process generally described as the oxidative burst. This event was first described in 1983 upon infection of potato (*Solanum tuberosum)* tubers with an incompatible race of *Phytophtora infestans* [5]. Since then, numerous studies have described the nature, kinetics, and localization of ROS production, mostly indicating the apoplast as the hotspot of ROS production (reviewed in [6]). In 1990, the role of calcium as a secondary messenger in the immunity response emerged [7,8], making both ROS and calcium the first-responders-on-scene, together with apoplast alkalinization and the activation of kinase modules [9] (Figure 1A). Subsequent series of pharmacological and genetic perturbation studies consolidated an upfront role for calcium fluxes and the necessity of a downstream oxidative burst for plant defence (measured by pathogen growth) [10-12]. Cytoplasmic calcium levels are kept low by sequestering free calcium ions in the apoplast and diverse subcellular stores. Within 1 min, pathogen recognition leads to a reversible release and uptake of calcium to the cytoplasm through calcium channels and pumps, respectively [13]. Originally, screens for mutant plants exhibiting altered defence response led to the identification of three lines (*dnd1, defence no death 1; dnd2/hml1, dnd2/HR-like lesion mimic 1*; and *cpr22, constitutive expresser of PR genes22)* that are linked to Ca^{2+} -permeable cyclic nucleotide-gated channels (CGNC2, CGNC4, and CGNC11/12, respectively). In addition, ionotropic glutamate receptor-like channels (iGluR), and calcium ATPases have also been implicated in plant immunity (for an in-depth review of calcium pumps and channels, refer to [14]; also see Figure 1A). The calcium signatures produced are stimulus-specific and are decoded by calcium-dependent protein kinases (CDPKs), whose deficiency in turn leads to impaired defence responses [15-17]. Although NADPH oxidases known as respiratory burst oxidase homologs (RBOHs) received the lion’s share of research interest, genetic evidence also indicated essential roles for apoplastic oxidative burst peroxidases for the PTI response. Peroxidase deficiencies caused decreased deposition of callose and expression of defense genes leading to higher pathogen susceptibilities [18].

A mix of ingredients: interplay between ROS and calcium signaling in the pathogen response

The oxidative burst is biphasic, with the first phase considered to be unspecific because it is also induced by other non-biotic stresses such as wounding, whereas the second, specific

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phase of prolonged ROS accumulation correlates with ETI and HR [19,20]. Increased ROS levels have a direct cytotoxic effect on pathogens, reinforce cell walls, and can serve as signaling molecules [6,21]. RBOHs are integral plasma membrane proteins that require nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FAD) cofactors for the apoplastic reduction of oxygen (O_2) to superoxide ($O_2^{\bullet-}$), which can subsequently be dismutated to H_2O_2 [22,23]. RBOHs are subject to calcium dependent post-translational regulation events during the immune response. In addition to two cytosolic EF-hand domains that allow direct regulation by intracellular calcium, RBOHs are phosphorylated at their N terminus by multiple CDPKs [15-17,24], and by a pathogen responsive calcineurin B-like protein (CBL) and CBL-interacting protein kinase (CIPK) module [25]. Recently, elicitor triggered phosphorylation of RBOHD by the PRR-associated Botrytis-induced kinase 1 (BIK1) was elegantly demonstrated to occur in parallel to – and was proposed to prime – calcium-dependent regulation of RBOHD [26,27] (Figure 1A). RBOH activity is crucial for the oxidative burst during pathogen response; however, its effect on pathogen growth is dependent on the plant–pathogen system [23]. Apoplastic peroxidases produce approximately half the amount of H_2O_2 produced during pathogen response [28]; hence, it raises the question if these peroxidases are also subject to calcium dependent regulation. Studying the interplay between peroxidases and RBOHs might conclusively clarify the role of the oxidative burst during pathogen response.

ROS–calcium interplay might also be important for systemic acquired resistance (SAR). RBOHs underlie systemic ROS signaling, which is a ROS wave that propagates from stimulated to nonstimulated tissue during several stress conditions [29]. CDPK5 was shown to phosphorylate RBOHD *in vivo* and *cdpk5* mutants displayed impaired SAR [16]. Similarly to ROS waves, root-to-shoot calcium waves were found to occur during salt stress [30] and iGLuR calcium channels propagated membrane depolarization (electrical signaling) from wounded leaves to distal leaves [31]. Therefore, it is tempting to speculate that the interplay of ROS and calcium waves, supported by RBOHD and CDPK5, form a cell-to-cell communication mechanism that transmits a long-distance (electrical) signal from infected tissue in order to establish SAR in distant non-infected tissue [32]. Ultimately, this could signal the ROS-responsive transcriptional co-regulator, nonexpressor of pathogenesis-related genes 1 (NPR1) that is central to the establishment of SAR [33,34] (Figure 1B).

Does a chloroplastic branch form a new calcium-ROS recipe?

Besides the importance of apoplastic ROS production within the oxidative burst, pioneering studies have also linked photosynthesis and chloroplastic ROS imbalances to plant immunity. High light-triggered photo-oxidative stress caused a systemic acquired acclimation reaction (SAA) that is very similar to SAR resulting in enhanced pathogen resistance [35,36]. Furthermore, lack of enhanced disease susceptibility 1 (EDS1), a central regulator of ETI and HR, was found to suppress cell death upon elicitation of chloroplastic $O_2^{\bullet-}$ and, based on gene expression analysis, was proposed to sense chloroplastic $O_2^{\bullet-}$ in order to initiate HR programmed cell death [37]. Central to ETI is a regulatory hub, composed of EDS1/phytoalexin deficient 4 (PAD4) and EDS1/senescence-associated gene 101 (SAG101) heterodimers, that is negatively regulated by lesion-simulating disease 1 (LSD1), thereby evoking salicylic acid (SA)-dependent SAR and immune responses [38,39].

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LSD1 inhibits the spread of cell death lesions mediated by the cysteine protease metacaspase 1 (AtMC1) [40] (Figure 1B). Notably, integration of ROS and SA signals by the LSD1/EDS1/PAD4 hub can lead to opposing effects in the HR programmed cell death, suggesting that it acts as a flexible spatiotemporal integration point leading to opposite reactions in infected and surrounding tissue [37].

Direct evidence for chloroplastic ROS production as a central player in immunity comes from a study describing delayed cell death during nonhost pathogen infiltration of tobacco (*Nicotiana tabacum*) plants overexpressing a chloroplast-targeted flavodoxin that prevents mainly $O_2^{\bullet -}$ and H_2O_2 formation. Remarkably, this only affected cell death without affecting defence gene expression [41]. Similarly, overexpression of a thylakoid membrane-bound ascorbate peroxidase (tAPX), which reduces H_2O_2 from the photosynthetic electron transport chain, diminished cell death during HR [42], although inducible repression of tAPX led to an increased expression of biotic stress marker genes and enhanced SA levels and response [43].

The previously mentioned studies focused on the role of photosystem I derived $O_2^{\bullet -}/H_2O_2$. However, singlet oxygen ($^1O_2$), mainly produced through photo-activation of O_2 by excited chlorophyll in photosystem II and to a minor extent in light harvesting complexes, has been implicated in immune responses as well [44,45]. Initially, this was based on overlapping transcriptional responses, the formation of HR cell death-like lesions, and the rise in oxylipin and jasmonic acid levels provoked by increased $^1O_2$ and by pathogen infection [46-48]. Recently, acclimation to light-induced $^1O_2$ production was shown to confer increased resistance to virulent *Pseudomonas* [48]. Instrumental to this work has been the *fluorescent in blue light* (*flu)* mutant, which overaccumulates photosensitive protochlorophyllide that results in chloroplastic $^1O_2$ production when transferred from dark to light, and the *flu* suppressors executer 1 and 2 (EX1 and EX2) that mediate $^1O_2$-induced programmed cell death [49,50]. Genetic screens, and the use of other mutants, chemicals, and natural ways of eliciting $^1O_2$ revealed a complex web of retrograde signaling underlying the observed gene expression reprogramming and cell death phenotypes with similarities to pathogen responses [45,49,51]. In addition, $^1O_2$ elicits a dose-responsive output, with high concentrations leading to oxidative damage of biomolecules and death by cytotoxicity, and lower concentrations leading to gene expression changes and acclimation to stress, such as pathogen attack [48,51].

While, for a long time, ROS dependent signaling events have been associated with the chloroplast, calcium fluxes and signaling in the chloroplast only recently gained attention (reviewed in [52-54]). Two independent studies reported that several PAMP-elicited calcium fluxes in the cytoplasm (peaking between 1 and 5 min) were rapidly followed by chloroplastic fluxes (maximum at 10–20 min) [55,56] (Figure 1A). Evidence has been provided that calcium signaling in the chloroplast affects both PTI and ETI and this pathway centers around the calcium sensing protein (CAS) [56]. Originally, CAS was considered an integral plasma membrane protein that is involved in the regulation of cytoplasmic calcium fluxes during exogenously applied calcium-induced stomatal closure [57]. However, it was subsequently demonstrated that CAS resides in the thylakoid membrane, while its mode of action remains unknown[58-60]. Treatment with the PAMP flg22 (a 22-amino acid moiety

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of the conserved N-terminal part of bacterial flagellin) led to a prolonged calcium flux (after a lag-period of 20 min) in the stroma of chloroplasts, which was partially abolished in *cas* mutants. Strikingly, CAS deficiency decreased resistance to both virulent and avirulent *Pseudomonas* strains. Based on these results and the similarity between CAS-dependent flg22-responsive genes and $^1O_2$ transcriptional signatures, the authors proposed that bacterial recognition may lead to a chloroplastic calcium flux, which subsequently leads to $^1O_2$ to signaling the nucleus for transcriptional reprogramming. Interestingly, CAS was found to be phosphorylated in a calcium-dependent manner, pointing to the potential involvement of chloroplastic kinases in this pathway [61]. We have performed a meta-analysis on publically available transcriptomics datasets monitoring gene expression after flg22 treatment together with ROS and calcium signaling related sets. This supports the hypothesis that CAS regulates flg22-induced immunity gene expression via $^1O_2$ and points out the possible involvement of retrograde signals, chloroplastic $O_2^{\bullet-}$ and the interplay with apoplastic H_2O_2 in the signaling pathway (Box 1 and Figure 2).

The question remains how chloroplastic calcium fluxes are mechanistically linked to downstream ROS signals. Interesting in this respect, is the recent observation that flg22 treatment leads to a rapid decrease in nonphotochemical quenching (NPQ; 20 min–1 h) [62]. NPQ is part of a series of protective mechanisms to prevent excessive energy transfer from light harvesting to enter the photosynthetic electron chain at photosystem II (PSII). This NPQ decrease was likely caused by a reduction in the protein levels of the PSII sub-unit protein S (PsbS), which is crucial for NPQ. A similar observation was made in the green algae *Chlamydomonas reinhardtii*, in which accumulation of the PsbS homologous protein (LHCSR3) and NPQ could be linked to CAS and calcium signaling [63]. This notion is further supported by a study in which stromal calcium flux in cryptogein-elicited cell suspension cultures correlated with increased chlorophyll fluorescence yield, which is a relative measure of PSII performance [55]. As a consequence, both an increased PSII function and a lowered NPQ lead to an increased amount of energy at PSII that can react with O_2 to form $^1O_2$ and subsequently affect transcriptional reprogramming during PTI (Figure 2D). On the contrary, increased ROS levels during ETI could have damaged PSII [64,65]. However, the plastoquinone pool, connecting PSII and photosystem I (PSI), becomes increasingly reduced (qP) [65]. Electrons reaching PSI can increasingly reduce O_2 in the stroma to $O_2^{\bullet-}$ (and subsequent dismutation to H_2O_2). This effect would be ameliorated when stromal electron acceptors (and ultimately CO_2 availability) are low, for example, when stomata are closed because of stomatal immunity [66,67] (Figure 2D). In addition to ROS, several retrograde signals could provoke nuclear transcriptional reprogramming [38,68]. An alternative mechanism for transcriptional reprogramming was recently reported in which light rapidly influences alternative splicing via a chloroplast retrograde signal [69]. It would be tempting to speculate that RNA processing could add a fast-acting post-transcriptional layer to defense gene expression.

Why does immune signaling branch via the chloroplast?

The biosynthesis pathways of several defense hormones originate in the chloroplast (e.g., SA and jasmonic acid, [70]) and, therefore, have to be integrated in the signaling responses during pathogen attack. In the proposed chloroplastic calcium–ROS branch, SA could take a

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prominent place, since $^1O_2$ [46], chloroplastic H_2O_2 [43], CAS [56], and light [71] were shown to induce the expression of *ISOCHORISMATE SYNTHASE1* and *2* genes (*ICS1* and *ICS2*), leading to increased SA levels. Interestingly, SA accumulation limits $O_2^{\bullet-}$ accumulation by a possible feedback loop in order to change the balance of ROS produced in the chloroplast [37]. However, additional factors may place the chloroplast at the center of immune responses.

Stomatal immunity or the closure of stomata upon pathogen recognition, has gained considerable attention during recent years and, similarly, is regulated by the same signaling pathways discussed in this opinion article[56,58,66,67]. It is tempting to speculate that plants have adopted the pathogen-triggered signaling pathways leading to stomatal closure, or parts of these, for defense purposes in other cell types.

Another obvious factor that may cause immune responses to branch through the chloroplast is light and the metabolic state of the cells. Light quality and quantity determine the severity of HR, which is tightly linked to the light-dependent production of ROS and SA in the chloroplast [41,65,71] and together with an increased SAR, ultimately lowers pathogen growth [71,72]. Because immune responses and plant growth need to be well-balanced [70], the cell is continuously monitoring its metabolic state, which depends foremost on chloroplast energy production and, thereby, light availability. In this scenario, the chloroplast may constitute a proverbial potentiometer that measures the quantity of light, thereby providing crucial feedback to the immune response on the metabolic state of the cell. The big overlap between differentially expressed genes (DEGs) between flg22, CAS, and CDPK experiments in the meta-analysis (Box 1) indicates that ‘chloroplast-dependent’ immune gene expression might play a synergistic or additive effect on gene dosage, thereby increasing severity of the PTI response and possibly HR. This would be reminiscent of the quantitative nature of defense gene expression during infection of *Arabidopsis* with different strains of *Pseudomonas*, where it was postulated that gene dosage, rather than switching on/off different gene sets, leads to the different outcome during compatible and incompatible interactions involving HR [73].

The recently discovered link between circadian rhythms and plant immunity might provide further insight into the chloroplastic branching of immunity. Defense responses are heightened at dawn, when pathogens are thought to be the most infectious or can enter leaves the most easily when stomata open for the day [74-77]. In this situation, the chloroplast might act more as a light switch than a potentiometer, to ensure correct gating of the first light at dawn. In order to ascertain the relative contribution of each proposed scenario, further investigation is needed.

Concluding remarks

In addition to the established link between chloroplasts and HR cell death, it seems that PTI is also heavily influenced by the chloroplast. The available data indicates that the chloroplastic branch of immunity, which involves a calcium–ROS relay influenced by light, is necessary early on in the infection stage for full resistance (summarized in Figure 2E). However, further research is needed to consolidate this view (see outstanding questions in

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Box 2). For example, the use of specific pharmacological inhibitors or genetic perturbations other than *cas* mutants, will be needed for confirmation. Therefore, elucidating the molecular mechanisms underpinning chloroplast calcium signaling and its link to ROS will be crucial. Spatiotemporal studies of the signaling that distinguish infected tissue from healthy surrounding tissue will be of crucial importance. Furthermore, the precise contribution of the chloroplastic branch to the well-defined cytosolic branch needs to be investigated; a synergistic cross-talk between the cytosolic and chloroplast routes seems plausible.

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Glossary

Compatible versus incompatible interaction
compatible interactions take place in susceptible hosts, which are not able to recognize the pathogen effectors and therefore do not mount an ETI response. This leads to colonization of the host tissues. By contrast, incompatible interactions involve recognition of the pathogen effectors by the host’s immune system, through direct or indirect interaction with NB-LRR proteins. This leads to ETI, which results in host resistance towards the pathogen, in most cases via an HR response.

Effector
proteins delivered by pathogens that modulate innate immunity and enable infection. Effectors can be secreted in the apoplastic space or into the cytoplasm of host cells through different secretion systems, where they target different subcellular compartments.

Effector-triggered immunity (ETI)
a second layer of plant defense which is initiated by recognition of effector proteins by NB-LRRs. ETI is an amplified version of PTI that often results in the induction of the hypersensitive response (HR) cell death.

Hypersensitive response (HR)
a plant-specific form of programmed cell death that typically accompanies and correlates with effector-triggered immunity (ETI) at the site of attempted pathogen invasion. HR is morphologically unique, involving cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling, vacuolization and chloroplast disruption during its final stages.

NB-LRRs
plant intracellular receptors of the nucleotide-binding and leucine-rich repeat domain class (NB-LRR). NB-LRRs that can directly or indirectly recognize effector proteins secreted into

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the host cell, activating effector-triggered immunity (ETI). Also known as disease resistance (R) proteins.

PAMP/MAMP-triggered immunity (PTI/MTI)
first layer of immunity activated by pathogen-associated molecular pattern (PAMP) recognition. Sometimes, this is also referred to as microbial-associated molecular pattern (MAMP) recognition. It involves a calcium burst, production of reactive oxygen species (ROS), callose deposition at the cell wall, activation of mitogen-activated protein kinase (MAPK) cascades, expression of defense-associated genes and production of ethylene.

Pathogen-associated molecular patterns (PAMPs)
conserved molecules common to pathogens that can be recognized by immune receptors in both plants and animals.

Pattern recognition receptors (PRRs)
host cell surface-localized receptors that can recognize PAMPs and initiate a signaling cascade leading to PAMP-triggered immunity (PTI).

Systemic acquired resistance (SAR)
defense mechanism that confers long-term protection against a broad spectrum of pathogens to the whole plant following an earlier, localized exposure. SAR requires salicylic acid and is characterized by increased expression of pathogenesis-related genes both locally, at the initial site of infection, and systemically, in uninfected tissue.

Virulent versus avirulent
a pathogen is termed avirulent if it contains an effector protein that can be recognized directly or indirectly by the host's immune system (via NB-LRR proteins) resulting in resistance. By contrast, a pathogen is considered virulent if the host is not able to recognize any of its effectors, and is therefore able to cause disease. Therefore, a given pathogen can be virulent in a host plant that lacks the cognate NB-LRR proteins, but avirulent in another host plant that contains the NB-LRR proteins that lead to direct or indirect recognition of the pathogen effector(s).

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Box 1

Case study – meta-analysis of flg22-induced transcriptional responses

To assess the interplay between ROS and calcium signaling and the possible role of chloroplasts during the early plant–pathogen interaction-induced transcriptional response, a meta-analysis of DNA microarray datasets monitoring gene expression after flg22 treatment (1 h after treatment, various concentrations) was performed and compared to CAS-regulated flg22-responsive genes [56], a calcium signaling dataset of CDPK-responsive genes [15], singlet oxygen ($^1O_2$) produced in the chloroplast, endogenously (photorespiratory) generated H_2O_2, and exogenously applied H_2O_2, chloroplastic $O_2^{\bullet -}$ generated by the addition of methyl viologen (MV) and β-cyclocitral (β-CC), a suspected chloroplast retrograde signal downstream of $^1O_2$ [78] (see Table 1 in main text).

Differentially expressed genes (DEGs) for each treatment are summarized in Table 2 in main text. Flg22, CAS, and $^1O_2$ datasets contain a significant amount of overlapping DEGs (see Figure 2A in main text, 100 DEGs), supporting a role for chloroplasts in immune signaling. This core of chloroplast-dependent flg22-responsive genes shows a significant overlap with β-CC (see Figure 2A in main text, 16 DEGs out of 100 core genes compared to 104 $^1O_2$/β-CC) pointing towards the possible involvement of this retrograde signal in the flg22 response. CDPK dependent genes show a significant overlap with flg22-responsive genes and surprisingly, with CAS dependent flg22-responsive genes (see Figure 2A and 2B in main text), which might indicate a synergistic effect on gene expression. Additional chloroplast retrograde signals to β-CC, or alternative routes next to $^1O_2$ mediated signaling exist and might play a role in the flg22 response, as revealed by the significant overlap between a flg22/CAS core and $O_2^{\bullet -}$ elicited by MV (see Figure 2C in main text). Furthermore, exogenous addition of H_2O_2 seems to overlap significantly with flg22. The significant overlap between H_2O_2 and the flg22/CAS core might point to a direct signaling role of exogenous H_2O_2 (possibly generated by RBOHD/F) to the chloroplast during the flg22 response. By contrast, endogenously generated H_2O_2 (*cat2)* does not overlap significantly with flg22/CAS (see Figure 2B in main text). The meta-analysis confirms a recent report showing that CAS and chloroplasts play a central role in the flg22 response (mitigated by $^1O_2$) [56] and points out the possible involvement of retrograde signals, chloroplastic $O_2^{\bullet -}$ and exogenous H_2O_2 in the signaling pathway.

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Box 2

Outstanding questions

- To what extent is the chloroplastic signaling branch shared between the PTI and ETI responses? What are the spatiotemporal differences in outcomes of ROS and calcium signaling?
- Is there organellar cross-talk for example with mitochondria and peroxisomes during plant immunity?
- What processes, other than transcriptional reprogramming and SA production, are affected by the chloroplastic signaling branch?
- Given that the described ROS and calcium signaling pathways are active during abiotic stresses, can the proposed chloroplast branch signaling scheme be generalized?

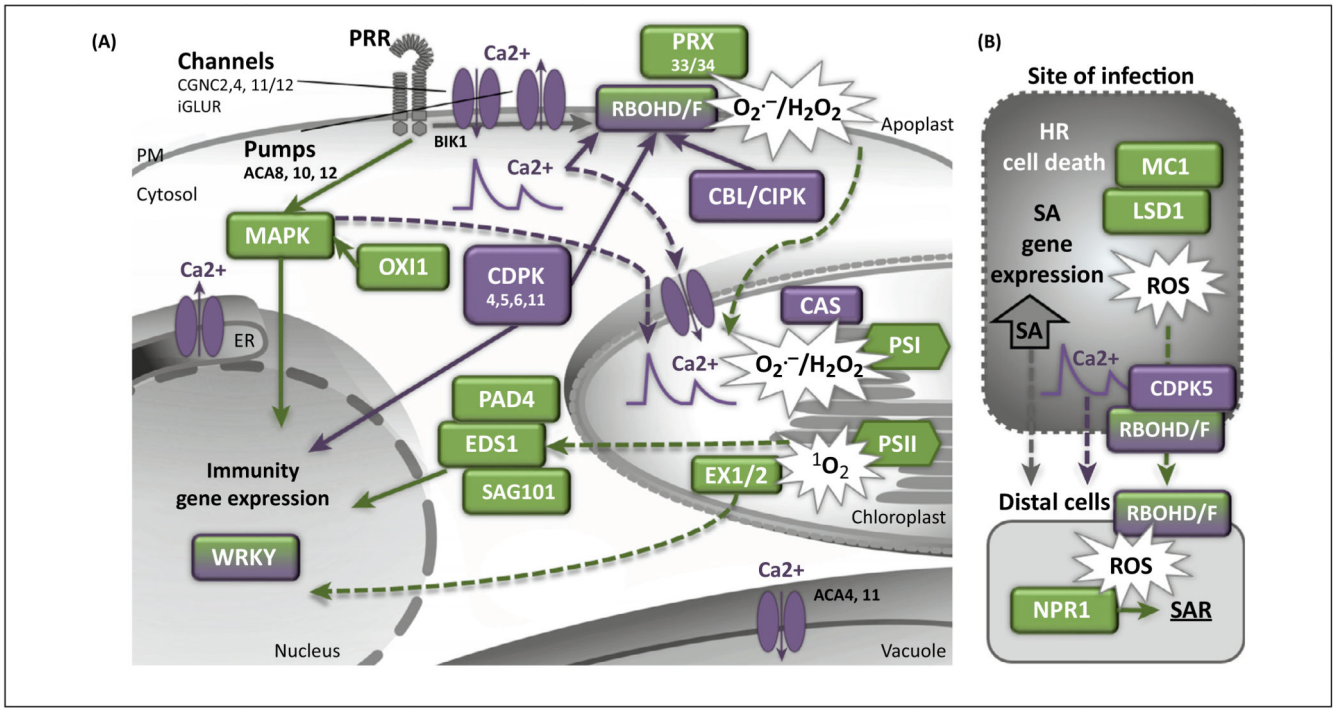

Figure 1.

Reactive oxygen species (ROS) and calcium signaling pathways during plant immunity. **(A)** A fast response is triggered upon pathogen-associated molecular pattern (PAMP) perception by pattern recognition receptors (PRRs) signaling the concerted action of calcium (Ca^{2+}) channels and transporters that generate a cytosolic Ca^{2+} flux (within 5 min after elicitation). Calcium-dependent protein kinases (CDPKs), upon activation by the Ca^{2+} flux, together with a mitogen-activated protein kinase (MAPK) cascade will trigger immunity gene expression in the nucleus, in which, for example, WRKY transcription factors play important roles. MAP kinases are regulated by the ROS sensory kinase oxidative signal-inducible 1 (OXI1). At the same time, Ca^{2+} flux and phosphorylation by Botrytis-induced kinase 1 (BIK1), CDPKs, and calcineurin B-like protein (CBL)/CBL-interacting protein kinase (CIPK) modules can enhance the activity of plasma membrane localized respiratory burst oxidase homologs (RBOHs) D and/or F (RBOHD/F) to produce apoplastic ROS ($O_2^{\bullet-}/H_2O_2$). Peroxidases 33 and 34 (PRX33/34) contribute to apoplastic ROS generation for the oxidative burst. Within 20 min of pathogen perception, a Ca^{2+} flux is generated in the chloroplast, which is regulated by the thylakoid associated calcium-sensing protein (CAS). Pathogen perception might be signaled to the chloroplast by a MAPK cascade, direct transfer of calcium from the cytosol to the chloroplast or H_2O_2 coming from the oxidative burst (or a combination thereof). Downstream retrograde signaling to the nucleus might involve the ROS $^1O_2$ (mainly generated by photosystem II [PSII]) and $O_2^{\bullet-}$ (mainly generated by photosystem I [PSI]). Executer1 and 2 (EX1/2) act downstream of $^1O_2$ to alter nuclear gene expression. The central immune regulator enhanced disease susceptibility 1 (EDS1) has been implicated downstream of chloroplastic $O_2^{\bullet-}$ and interacts with phytoalexin deficient 4 (PAD4) and Senescence-Associated gene 101 (SAG101) as heterodimers to alter nuclear immunity gene expression. **(B)** A later response to pathogen

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infection involves the ROS sensory protein lesion simulating disease 1 (LSD1), which was postulated to inhibit spread of cell death lesions mediated by metacaspase 1 (MC1) during hypersensitive response (HR) type cell death (indicated by darkened color and membrane perforations). Enhanced immunity gene expression leads to increase of the immune hormone salicylic acid (SA) in the chloroplast (indicated by an upwards pointing arrow). A spreading SA signal, calcium fluxes, and ROS signal (putatively via RBOHD/CDPK5 relay) could signal to the ROS sensory protein *Arabidopsis* nonexpressor of pathogenesis-related genes 1 (NPR1) to activate systemic acquired resistance (SAR) in distal cells of the site of infection. Proteins connected to Ca^{2+} signaling are denoted in purple. Proteins connected to ROS signaling are shown in green. Hypothetical connections are broken. Abbreviations: CGNC, Ca^{2+}-permeable cyclic nucleotide-gated channel; ER, endoplasmic reticulum; iGluR, ionotropic glutamate receptor-like channels; PM, plasma membrane; ACA4, 8, 10, 11, 12, autoinhibited calcium-ATPase 4, 8, 10, 11, 12.

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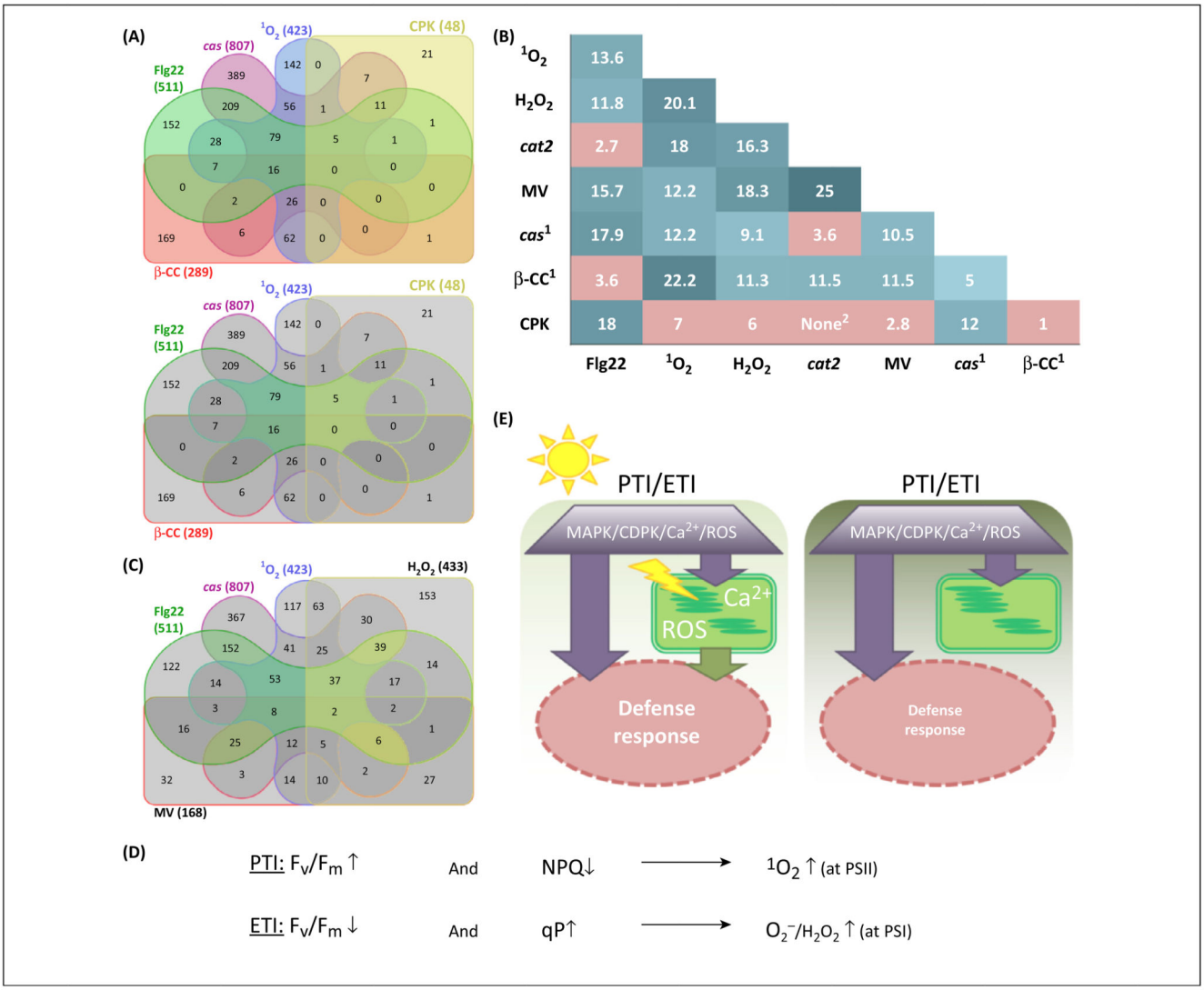

Figure 2.

Meta-analysis of flg22-induced transcriptional responses underscores a working hypothesis that pathogen-triggered immunity (PTI) includes a putative chloroplast signaling branch. **(A)** Overlap of flg22, *cas*, $^1O_2$, CPK, and β-cyclocitral (β-CC) transcriptomic datasets. Total number of differentially-expressed genes (DEGs) per elicitor is given between brackets. The bottom image in (A) is the same as the top image, but highlights the core overlap between flg22, *cas*, and $^1O_2$. Venn diagrams were constructed using publicly available software (http://bioinformatics.psb.ugent.be/webtools/Venn/, VIB/UGent Bioinformatics & Evolutionary Genomics, Ghent, Belgium). **(B)** The statistical significance of overlaps was assessed using the Fisher exact test (99% confidence interval). The ratio between observed and expected number of overlapping DEGs is displayed, a higher number (dark blue) means a higher statistical significance of the overlap (P value). Values in red were not significant. **(C)** Overlap of flg22 and *cas* (highlighted) with the reactive oxygen species (ROS) $^1O_2$, exogenous H_2O_2 and methyl viologen (MV)-generated $O_2^{\bullet -}/H_2O_2$. Note the difference in elicitors to (A) (H_2O_2 and MV, written in black). **(D)** Reported chlorophyll fluorescence

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measurements and hypothetical chloroplast ROS formation during PTI and effector-triggered immunity (ETI) responses [55,62,64,65]. Abbreviations: Fv/Fm, chlorophyll fluorescence parameter for the maximum efficiency at which absorbed light is used for reduction of the first electron acceptor of PSII; NPQ, a mechanism to dissipate excess excitation energy as heat in order to prevent overreduction of the photosynthetic electron chain by excess light; qP, photochemical quenching or the amount of energy being transferred to the photosynthetic electron transport chain via plastoquinone; PSII, photosystem II is the first protein complex of the photosynthetic electron transport chain at the thylakoid membrane; PSI, photosystem I is the second photosystem and is connected to PSII by the plastoquinone pool and the cytochrome b6f protein complex. **(E)** Schematic overview of a chloroplastic signaling branch composed of Ca^{2+}- and ROS-signaling, parallel to the established cytoplasmic signaling branch, composed of mitogen-activated protein kinase (MAPK)-, calcium-dependent protein kinases (CDPK)-, and Ca^{2+}-signaling, that depending on light availability, will influence the extent of immunity gene expression during PTI and ETI.

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Table 1

Meta-analysis datasets[a]

Elicitor	Experiment	Material	Platform	Rep.	Norm.	Data	Refs
Flg22							
	1 h 1 μM flg22	Mesophyll protoplasts	ATH1	2	gcRMA	GSE16472	[15]
	1 h 2 nM flg22	Seedling, 8 days	ATH1	2	gcRMA	GSE17382	[15]
	1 h 1 μM flg22	Seedling, 10 days	ATH1	3	gcRMA	Obtained	[79]
	1 h 100 nM flg22	Leaf discs, 5 weeks	ATH1	3	gcRMA	GSE17464	–
	1 h 100 nM flg22	Leaf discs, 5 weeks	ATH1	3	gcRMA	GSE17479	–
	1 h 1 μM flg22	Leaf, 5 weeks	ATH1	3	gcRMA	GSE5615	–
$^1O_2$							
Chl	2 h Dark-light shift (100 μE m^{-2} s^{-1}) – *flu* versus wild type	Rosette, 3 weeks	ATH1	2	gcRMA	GSE10812	[80]
	0.5 h Dark–light shift (100 μE m^{-2} s^{-1}) – *flu* versus wild type	Rosette, 3 weeks	ATH1	2	gcRMA	GSE10509	[81]
	2 day SD (1000 μE m^{-2} s^{-1}) – *ch1* versus wild type	Rosette, 8–5 weeks	CATMA	3	LOESS *	GSM845703	[82]
H_2O_2 – $O_2^{\bullet-}$							
Per[b]							
	[CO_2] shift (4500 to 400 μl l^{-1}) – 2.4 days SD versus 0 h	Rosette, 5 weeks	ATH1	3	gcRMA	GSE27985	[83]
	RGCL assay (CO_2 limitation) – 24 h versus 0 h	Seedling, 2 weeks	ATH1	3	gcRMA	In-House	–
	8 h HL shift (140–1800 μE m^{-2} s^{-1}) – *cat2* versus wild type	Rosette, 6 weeks	ATH1	2	gcRMA	In-House	[84]
Apo[b]	10 mM H_2O_2 – 24 h versus 0 h treated	Seedling, 9 days	ATH1	3	gcRMA	In-House	–
	20 mM H_2O_2 spray – 3 h treated versus control	Seedling, 2 weeks	ATH1	3	gcRMA	GSE41136	[85]
	5 mM H_2O_2 – 1 h treated versus Control	Seedling, 5 days	ATH1	3	gcRMA	GSE5530	[86]
Chl[b]	50 μM MV – 2 h treated versus control	Seedling (aerial), 2 weeks	ATH1	2	gcRMA	E-ATMX-28	[87]
	25 μM MV – 3h treated versus control	Seedling, 2 weeks	ATH1	2	gcRMA	GSE41963	–
	10 μM MV – 12 h treated versus control	Seedling (aerial), 2.5 weeks	ATH1	3	gcRMA	ME00340	–
CAS							
	2 h 1 μM flg22 – *cas* versus wild type	Seedling, 2 weeks	Arab4	2	LOESS **	Paper	[56]
β-CC							
	4 h 50 μl β-CC	Rosette, 4 weeks	CATMA		LOES *	GSE33963	[78]
CDPK							
	CDPK overexpression – 6 h post-transfection	Mesophyll protoplasts	ATH1	2	gcRMA	GSE16471	[15]

*Adjusted *P* value <0.05.

** *P* value <0.01.

[a] Cross-platform comparison was avoided as much as possible by choosing ATH1-platforms as input, in addition only datasets based on a minimum of two biological replicates were included. Robust Multiarray Average (gcRMA; [80-90]) was conducted using the affy package of R/Bioconductor [91] for normalization. A grouped *t*-test was performed and only log fold changes with a *P* value <0.001 were retained. In the exceptional cases that datasets from other platforms had to be included [56,78,82], the DEGs were extracted from their respective publications.

[b] Abbreviations: Apo, apoplast; Chl, chloroplast; Per; peroxisome; Rep., replicates; Norm., normalization method; SD, short days; flu, fluorescent in blue light; ch1, chlorina 1; ATH1, GeneChip® *Arabidopsis* ATH1 Genome Array (Affymetrix); CATMA, Complete *Arabidopsis* Transcriptome MicroArray; Arab4, *Arabidopsis* v4 2 color microarray (Agilent Technologies); RGCL, restricted gas continuous light; HL, high light; flg22, flagellin 22, MV, methyl viologen; β-CC, β-cyclocitral; CDPK, calcium dependent protein kinase; CAS, calcium sensing protein.

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Table 2

Overview of DEGs per treatment[a]

Elicitor	Cut-Off	Upregulated	Downregulated	Total
Flg22	3/6 Exp	511	75	586
$^1O_2$	2/3 Exp	423	87	510
H_2O_2				
cat2	2/3 Exp	133	2	135
H_2O_2	2/3 Exp	433	60	493
MV	2/3 Exp	168	15	183
CAS	Paper	398	807	1205
β-CC	Paper	289	86	375
CPK	1/1 Exp	48	0	48

[a]Probes with a minimum fold change of 2 (or −2) were designated as DEGs.