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REVIEW PAPER

Chloroplasts at work during plant innate immunity

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Abstract

The major role played by chloroplasts during light harvesting, energy production, redox homeostasis, and retrograde signalling processes has been extensively characterized. Beyond the obvious link between chloroplast functions in primary metabolism and as providers of photosynthesis-derived carbon sources and energy, a growing body of evidence supports a central role for chloroplasts as integrators of environmental signals and, more particularly, as key defence organelles. Here, we review the importance of these organelles as primary sites for the biosynthesis and transmission of pro-defence signals during plant immune responses. In addition, we highlight interorganellar communication as a crucial process for amplification of the immune response. Finally, molecular strategies used by microbes to manipulate, directly or indirectly, the production/function of defence-related signalling molecules and subvert chloroplast-based defences are also discussed.

Key words: Chloroplast, interorganellar signalling, microbial effector, plant immunity, pro-defence molecule, retrograde signalling, stromule.

Introduction

The chloroplast is a vital component of photosynthetic cells in cyanobacteria, algae, and higher plants, since it is the organelle in which photosynthesis takes place. Chloroplasts are large plant cell organelles bounded by a double-celled composite membrane with an intermembrane space, called the chloroplast envelope. In addition to the inner and outer membranes, chloroplasts have a third internal membrane system, known as the thylakoid membrane, which is extensively folded. This thylakoid membrane divides the stroma, which lies inside the envelope but outside the thylakoid membrane, and the thylakoid lumen (Cooper, 2000). The thylakoid membrane contains chlorophyll and other pigments responsible for capturing light energy. Although photosynthesis is the major function of the chloroplast, its roles clearly extend further than converting light energy into chemical energy.

It seems obvious that plants undergo an increased demand for photosynthesis during the interaction with pathogens, as the biosynthesis of pro-defence molecules and, more generally, the induction of defence responses requires energy that is provided through photosynthesis (Hammerschmidt, 1999; Swarbrick and Lefert, 2006). Moreover, virulent pathogens feed on plant carbon compounds, and some of them are able to use plant transporters of the SWEET family to promote sugar efflux, further increasing photosynthesis demand in host cells (Chen *et al.*, 2010). However, instead of increased photosynthesis, several reports have revealed suppression of photosynthetic functions in infected plants, perhaps reflecting an active plant response to shut down carbon availability and limit pathogen growth or to favour the establishment of defence over other physiological processes, including photosynthesis, during pathogen attack. Along these lines, the chloroplast is emerging as a very dynamic signalling compartment that is able to sense perturbations (biotic and abiotic stresses) at the subcellular level and to integrate a

multitude of intracellular signals in order to communicate those perturbations to other organelles (Padmanabhan and Dinesh-Kumar, 2010; Bobik and Burch-Smith, 2015). The chloroplast, together with the nucleus, cell membrane, and endoplasmic reticulum (ER), plays a critical role during the establishment of plant immunity against microbial attack and, in this context, the importance of efficient interorganellar signalling to achieve a synchronized whole-cell response during plant defence responses is becoming increasingly evident (Padmanabhan and Dinesh-Kumar, 2010; Nomura *et al.*, 2012; Bobik and Burch-Smith, 2015; Caplan *et al.*, 2015). The orchestration of this intracellular signalling is achieved by the action of the chloroplast as a receiver, an integrator, and a transmitter of specific signals that co-ordinate expression of nuclear and plastid genomes in order to sustain homeostasis.

Plants are sessile organisms lacking an adaptive immune system. Nonetheless, they have developed a multilayered defence system that effectively protects them from a wide range of pathogens, including bacteria, viruses, and fungi. The immune system of plants can be divided into two layers of defence responses. The first line of defence is activated by the detection of conserved pattern-associated molecular patterns (PAMPs), by pattern-recognition receptors (PRRs), leading to PAMP-triggered immunity (PTI), a basal immune status effective against a broad spectrum of pathogens (Nicaise *et al.*, 2009; Macho and Zipfel, 2014). To combat the defence strategies of the plant, host-adapted pathogens secrete various effector molecules that manipulate host defence responses and facilitate colonization (Dangl and Jones, 2006; Göhre and Robatzek, 2008; Dodds and Rathjen, 2010; Trotta *et al.*, 2014). In the course of plant–pathogen co-evolution, plants have evolved resistance (R) proteins that detect pathogen effectors, either directly or via the effect that they produce on a plant target, then activating effector-triggered immunity (ETI) (Dodds and Rathjen, 2010; Dangl *et al.*, 2013; Stuart *et al.*, 2013).

A hallmark of ETI is the hypersensitive response (HR), a localized form of programmed cell death (PCD) in the vicinity of the infection site that relies on a burst of reactive oxygen species (ROS) in chloroplasts (Zurbriggen *et al.*, 2010). Moreover, the chloroplast is involved in the synthesis of important mediators of plant immune responses, such as the hormones salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA), and secondary messengers including calcium and ROS (León and Sánchez-Serrano, 1999; Wildermuth *et al.*, 2001; Nambara and Marion-Poll, 2005; Torres *et al.*, 2006; Kozuleva *et al.*, 2011; Wasternack and Hause, 2013; Bobik and Burch-Smith, 2015; Stael *et al.*, 2015). In addition to hosting biosynthesis of defence-related molecules, recent reports highlight the importance of the chloroplast in the sensing of signals and in the amplification of downstream signalling during PTI and ETI (Nomura *et al.*, 2012; Caplan *et al.*, 2015). Given the crucial role played by chloroplasts in the orchestration of defence signalling, it is thus not surprising that microbes target chloroplast-related functions, and in some cases chloroplasts themselves, in their attempt to subvert host defences and promote virulence.

Here we review the central role of chloroplasts in the production and delivery of pro-defence molecules, as well as in the integration of biotic stress signals and in the amplification of downstream signalling. Molecular strategies used by pathogens to hijack chloroplast functionality, directly or indirectly, are also discussed.

The chloroplast: a major production site of pro-defence molecules

Chloroplasts play a central role in plant immunity by hosting biosynthesis of several key defence-related molecules, including hormones and secondary messengers such as calcium and ROS (Fig. 1).

SA biosynthesis and accumulation are tightly regulated since constitutive SA accumulation has negative impacts on plant fitness (Ishihara *et al.*, 2008; Pajerowska-Mukhtar *et al.*, 2012; Chandran *et al.*, 2014). SA biosynthesis is triggered during PTI and ETI, upon recognition of PAMPs and effectors, respectively (Mishina and Zeier, 2007). Plants synthesize SA through two distinct enzymatic pathways: the isochorismate (IC) and the phenylalanine ammonia-lyase (PAL) pathways. Both pathways commonly utilize chorismate, the end-product of the shikimate pathway, to produce SA, but the IC pathway, which is operative in plastids, is the predominant source of both basal and pathogen-induced SA production in Arabidopsis (Dempsey *et al.*, 2011). In chloroplasts, IC synthases catalyse the conversion of chorismate into IC (Wildermuth *et al.*, 2001; Strawn *et al.*, 2007; Garcion *et al.*, 2008) that is further converted to SA (Dempsey *et al.*, 2011). SA is a key signalling molecule in resistance against biotrophic and hemibiotrophic pathogens (Pieterse *et al.*, 2012), although it has also been shown to be involved in the response to necrotrophic pathogens (Glazebrook, 2005). SA plays a primary role not only in local PTI and ETI responses (Pieterse *et al.*, 2012; Seyfferth and Tsuda, 2014; Tanaka *et al.*, 2015), but also in systemic defence signalling during systemic acquired resistance (SAR), a long-lasting and broad-spectrum induced resistance to secondary infection that follows the onset of local defences (Vlot *et al.*, 2009; Spoel and Dong, 2012). In addition, SA is a central regulator of defence responses by crosstalk with other hormone signalling pathways in order to fine-tune plant responses with the minimal fitness cost (Vlot *et al.*, 2009; Thaler *et al.*, 2012).

JA is a lipid-derived hormone whose biosynthesis begins in the chloroplast but is completed in the peroxisome. JA biosynthesis is initiated by the release of α-linolenic acid from galactolipids of plastidial membranes and followed by the consecutive action of plastid-localized lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) to render the precursor molecule *cis*-OPDA [*cis*-(+)-12-oxophytodienoic acid]. *cis*-OPDA is then transported to peroxisomes for subsequent reduction and β-oxidation, giving rise to the final product, (+)-7-iso-JA. After being released into the cytosol, (+)-7-iso-JA is conjugated to the amino acid isoleucine to form the bioactive form of the hormone, (+)-7-iso-JA-Ile (JA-Ile) (Wasternack and Hause, 2013). In

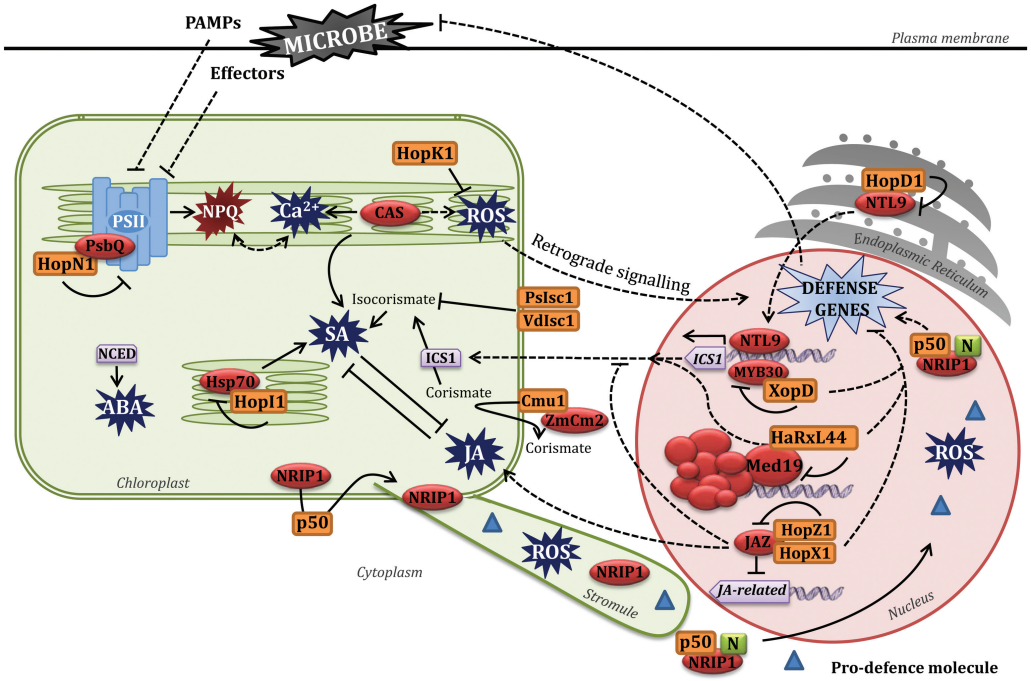

Fig. 1. The chloroplast plays a central role during plant immunity. PAMP perception leads to reduced PSII activity and subsequent reduction in NPQ. The chloroplast-localized protein CAS is involved in stromal Ca^{2+} transients, which have been proposed to be linked to NPQ and to a ROS burst. ROS production induces nuclear defence gene expression by retrograde signalling, which restricts pathogen growth. Some effectors are able to inhibit photosynthetic CO_2 assimilation, thus blocking the ROS burst and favouring pathogen proliferation. Interaction of the effector HopN1 with the PSII protein PsbQ blocks ROS production. Major steps of biosynthesis of defence-related hormones SA, JA, and ABA occur in the chloroplast. Chloroplastic pro-defence signal biosynthesis promotes stromule formation. The viral effector p50 induces relocalization of chloroplastic NRIP1 and pro-defence molecules to the nucleus where the R protein N is able to induce defence gene expression. Cytoplasmic interaction between the chorismate mutase effector protein Cmu1 and the host chorismate mutase ZmCm2 reduces chorismate availability for SA biosynthesis in the chloroplast. The effectors PsIsc1 and VdIsc1 hydrolyse isochorismate, thus perturbing SA homeostasis. Interaction of HopI1 with chloroplastic Hsp70 blocks SA production. HopK1 is also able to block ROS production, although its target(s) remain(s) unknown. The effector XopD blocks MYB30-mediated nuclear induction of *ICS1* expression. Interaction of HopD1 with NTL9 in the ER may result in reduced NTL9-mediated induction of *ICS1*. HaRxL44 induces Med19a proteasomal degradation and thus repression and induction of SA and JA signalling, respectively. HopZ1a and HopX1 induce degradation of JA signalling repressors, thus enhancing JA and repressing SA signalling. Effectors are indicated as orange rectangles and plant proteins as red ellipses. See the text for details.

contrast to SA, the production of JA is induced after the attack of necrotrophic pathogens, nematodes, and herbivorous insects (reviewed in Pieterse *et al.*, 2012; Wasternack and Hause, 2013; Campos *et al.*, 2014). Since the first described example in tomato, antagonism between SA and JA signalling pathways has been uncovered in numerous plant species and revealed that increased resistance against biotrophs often correlates with enhanced susceptibility to necrotrophs, and vice versa (van Wees *et al.*, 1999; Spoel *et al.*, 2003; Grant and Lamb, 2006; Gimenez-Ibanez and Solano, 2013). This active interplay between SA and JA contributes to optimize the defence response by prioritizing one signalling pathway over the other, depending on the invading pathogen (Kunkel and Brooks, 2002; Bostock, 2005; Verhage *et al.*, 2010).

ABA is derived from C_{40} epoxycarotenoid precursors, also called xanthophylls, in the plastid (Nambara and Marion-Poll, 2005). Cleavage of *cis*-isomers of xanthophylls by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) represents a first key step towards ABA biosynthesis to form a C_{15} product, xanthoxin. Xanthoxin is then converted to ABA by a two-step reaction in the cytosol (Nambara and Marion-Poll, 2005). ABA is well known as a major stress-related plant hormone in the abiotic stress response, particularly in response to drought. In addition, ABA can also act as a positive or a negative regulator of plant defence depending on the interaction (Mauch-Mani and Mauch, 2005; Asselbergh *et al.*, 2008; Fan *et al.*, 2009; Ton *et al.*, 2009; Kazan and Lyons, 2014). Negative interactions of ABA with major defence-related phytohormones, including SA and JA, have been described (Yasuda *et al.*, 2008; de Torres Zabala *et al.*, 2009; Sánchez-Vallet *et al.*, 2012). Similarly, our view on other chloroplast-derived phytohormones changed recently as a role for phytohormones, such as SA or JA with important roles in defence against pathogens, additionally emerged in the context of abiotic stress responses. This aspect is discussed in the review article by Kmiecik and co-workers in this special issue (Kmiecik *et al.*, 2016).

In addition to their role in the biosynthesis of defence-related hormones, chloroplasts are also important sites of intracellular Ca^{2+} storage in plant cells (Stael *et al.*, 2012*a*). Extracellular PAMP signals are rapidly relayed to chloroplasts during the early stages of immune signalling (Stael *et al.*, 2015). Indeed, perception of flg22, chitin, cryptogein, or oligogalacturonides generates a transient Ca^{2+} increase in the chloroplast stroma within a few minutes (Manzoor *et al.*, 2012; Nomura *et al.*, 2012). The chloroplast-resident, Ca^{2+}-binding

protein CALCIUM-SENSING RECEPTOR (CAS) plays a critical role in connecting chloroplasts to cytoplasmic–nuclear immune responses triggered during both PTI and ETI and is involved in the regulation of SA biosynthesis. Ca^{2+}-dependent phosphorylation of CAS was previously demonstrated (Stael *et al.*, 2012*b*). Potential links between non-photochemical quenching (NPQ) and Ca^{2+} signalling have been suggested (Petroutsos *et al.*, 2011; Manzoor *et al.*, 2012). Gene expression analysis after flg22 elicitation further revealed that CAS is required for the down-regulation of nuclear-encoded photosynthesis-related genes and up-regulation of defence gene expression through ROS-mediated retrograde signalling to the nucleus (Nomura *et al.*, 2012). Indeed, in addition to the primary apoplastic ROS production that is rapidly triggered after pathogen perception, the chloroplast is an essential provider of ROS during the initiation and promotion of cell death during the HR (Straus *et al.*, 2010; Chaouch *et al.*, 2012; Shapiguzov *et al.*, 2012). A recent study suggested that ROS production may restrict bacterial multiplication of non-pathogenic *hrp* mutant bacteria and potentially be the target of effector proteins from wild-type bacterial strains (Mitchell *et al.*, 2015). Reduction of O_2 by the photosynthetic electron transport chain in chloroplasts can result in the formation of a series of reduced forms of O_2, or ROS, that include the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH\cdot$). ROS also include singlet oxygen ($^1O_2$), which is generated by energy transfer from other molecules, mainly from an excited chlorophyll triplet state (Pospisil, 2011). Analysis of the chloroplast proteome after PAMP treatment showed reduced accumulation of six photosynthetic proteins including PsbS, a photosystem II (PSII) subunit that is involved in dissipation of excessive light energy by NPQ (Göhre *et al.*, 2012). Weaker accumulation of the PsbS protein correlates with reduced NPQ, which is linked to accumulation of excited electrons and ROS production in the chloroplast (Hideg *et al.*, 2008). In agreement with these findings, recent global transcriptome studies show that PAMP perception leads to rapid suppression of nuclear-encoded chloroplast-targeted genes (NECGs), in particular down-regulation of photosynthetic gene expression, which is followed by production of photosynthesis-derived ROS in the chloroplast (de Torres Zabala *et al.*, 2015; Lewis *et al.*, 2015). Indeed, early PAMP-triggered transcriptional responses (between 0 and 2 h post-inoculation) were found to be related to chloroplast-related functions, including ROS production, SA synthesis, and photosynthesis (Lewis *et al.*, 2015). Interestingly, a recent study showed that light is required for both up- and down-regulation of genes induced and repressed, respectively, after flg22 treatment, as well as for SA accumulation (Sano *et al.*, 2014). Moreover, photosynthetic electron flow appears to play a central role in controlling the light-dependent expression of flg22-inducible defense NECGs (Sano *et al.*, 2014). Finally, induction of defence gene expression following ROS-mediated retrograde signalling correlates with restriction of bacterial proliferation although a direct link among the three processes remains to be formally demonstrated (Göhre and Robatzek, 2008; Nomura *et al.*, 2012; de Torres Zabala *et al.*, 2015; Lewis *et al.*, 2015).

Stromules function as molecular highways to transduce pro-defence molecules from choloroplasts to the nucleus

In tobacco, recognition of the 50 kDa helicase domain of *Tobacco mosaic virus* (TMV) replicase (p50) by the NBL protein N leads to HR-mediated cell death and plant resistance. Caplan and co-workers previously showed that this recognition event requires the chloroplastic protein NRIP1 (N Receptor Interacting Protein1) (Caplan *et al.*, 2008). Intriguingly, NRIP1 was reported to relocalize from chloroplasts to the cytoplasm and nucleus of TMV-infected tobacco cells. Although the mechanism involved in NRIP1 relocalization was not described, the authors suggested the involvement of stromules in NRIP1 nuclear import during N-mediated defence (Caplan *et al.*, 2008). Stromules (stroma-filled tubules) are dynamic structures extending from the surface of all plastid types, including etioplasts, leucoplasts, amyloplasts, and chromoplasts (Natesan *et al.*, 2005).

In a recent work, Caplan and collaborators confirmed the formation of highly dynamic tubular projections from chloroplasts during N-mediated viral defence in *Nicotiana benthamiana* (Caplan *et al.*, 2015). Similarly, stromule formation was observed in response to bacteria-triggered ETI in Arabidopsis, suggesting that stromule induction may be a general response during the immune response against bacteria and viruses (Caplan *et al.*, 2015). Induction of stromules was observed both within the infection site and along the borders of sites where HR was developing, suggesting that pro-defence signalling molecules may play a role in cell to cell signalling during stromule formation. Indeed, exogenous application of H_2O_2 and SA induced the formation of stromules, thus supporting the idea that pro-defence molecules may act as signals for stromule induction.

It was suggested that stromules may play a role in facilitating the transport of molecules among organelles by increasing the surface area of exchange and bringing plastids into close proximity with other organelles (Hanson and Sattarzadeh, 2011, 2013). Indeed, association of stromules with the plasma membrane, mitochondria, and nuclei has been described (Hanson and Sattarzadeh, 2011; Schattat *et al.*, 2011*a*, *b*), suggesting that stromules may allow the exchange of proteins and metabolites between plastids and other subcellular compartments (Kwok and Hanson, 2004; Brunkard *et al.*, 2015). Remarkably, Caplan and co-workers showed that stromules develop abundant connections with nuclei during HR-inducing ETI, tightly tethering the chloroplast outer membrane and the nuclear envelope (Caplan *et al.*, 2015). These associations of chloroplasts and nuclei correlate with increased nuclear accumulation of NRIP1 that moves from chloroplasts and accumulates in nuclei during immune signalling. By monitoring H_2O_2 levels after induction of the immune response, the authors showed that, following a burst of H_2O_2 production in the chloroplast, H_2O_2 moves from the chloroplast to the nucleus through chloroplast to nucleus connections (Caplan *et al.*, 2015).

Overexpression of the targeting peptide of CHUP1 (Chloroplast Unusual Positioning1), an actin-binding

domain-containing protein that is targeted to the chloroplast outer envelope, results in strong reduction of stromule formation under HR conditions (Caplan *et al.*, 2015). In contrast, *CHUP1* silencing and knockout results in constitutive formation of stromules and enhanced HR. These results are in agreement with previous observations that stromules extend through actin microfilaments (Kwok and Hanson, 2004) and underline the role of stromules in enhancing defence-related HR.

Taken together, these results demonstrate that pathogen perception triggers the formation of stromules, which involves dynamic morphological changes at the chloroplast envelope. Moreover, stromules mediate transport of pro-defence molecules into the nucleus, and probably other subcellular compartments, thereby contributing to the amplification of defence signalling (Fig. 1). Finally, abiotic stress conditions also trigger the formation of stromules, and this is reviewed by Kmiecik and co-workers in another article of this special issue (Kmiecik *et al.*, 2016).

Microbial effectors are able to interfere with the production/function of pro-defence molecules in the chloroplast

Consistent with the importance of a balanced production of pro-defence molecules during plant–pathogen interactions, pathogens have developed sophisticated molecular mechanisms to subvert their biosynthesis and subsequent signalling for their own benefit (Denancé *et al.*, 2013; Kazan and Lyons, 2014). The central role of hormones during plant–pathogen interactions is highlighted by the significant number of pathogenic microbes that are able to produce hormones or hormone-mimicking molecules to disturb hormone homeostasis and cause disease (Robert-Seilaniantz *et al.*, 2011; Denancé *et al.*, 2013). Since hormone production largely depends on chloroplast-based metabolic pathways, in this section we discuss effectors that are able to interfere with the production and/or function of phytohormones even without being directly targeted to plastids (Fig. 1).

The biotrophic fungus *Ustilago maydis* secretes the effector Cmu1 into the cytoplasm of maize cells. Consistent with the finding that Cmu1 is an active chorismate mutase, an isomerase that converts the SA precursor chorismate into prephenate, leaves infected with an *U. maydis cmu1* mutant showed increased SA accumulation (Djamei *et al.*, 2011). Interaction of Cmu1 with the maize chorismate mutase ZmCm2 in the cytoplasm of host cells was proposed to enhance the flow of chorismate from the plastid to the cytosol, thus reducing chorismate availability for SA biosynthesis in the chloroplast. Indeed, *cmu1* mutants are impaired in virulence, highlighting the role of SA in maize resistance to *U. maydis* (Djamei *et al.*, 2011; Djamei and Kahmann, 2012). Similarly, the effectors PsIsc1 and VdIsc1 from the unrelated filamentous pathogens *Phytophthora sojae* and *Verticillium dahliae,* respectively, are secreted isochorismatases able to hydrolyse isochorismate in host cells, thus modulating SA biosynthesis and suppressing host immunity (Liu *et al.*, 2015).

Other effectors are able to target the transcriptional control of SA production directly. For example, the Arabidopsis transcription factor MYB30 is involved in an amplification loop modulating *ICS1* expression, and thus SA biosynthesis, which in turn modulates defence-related HR-mediated cell death after bacterial infection (Raffaele *et al.*, 2006). Targeting of the nuclear transcription factor MYB30 by the effector XopD from *Xanthomonas campestris* leads to reduced *ICS1* expression, thus compromising plant resistance and HR (Canonne *et al.*, 2011). The *Pseudomonas syringae* pv. *tomato (Pst)* effector HopD1 interacts with and suppresses the transcriptional activation of the ER-resident transcription factor NTM1-LIKE 9 (NTL9), thus contributing to suppression of host ETI (Block *et al.*, 2014). A recent report showed that NTL9 activates transcription of *ICS1*, which suggests that HopD1-mediated inhibition of NTL9 may affect *ICS1* activation, and thus SA production, during immune signalling (Zheng *et al.*, 2015). The effector HaRxL44 from the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) interacts with the subunit 19a of the Mediator complex (Med19a) that positively regulates resistance to *Hpa* (Caillaud *et al.*, 2013). HaRxL44 leads to Med19a proteasomal degradation and enhanced susceptibility to *Hpa* through attenuation of SA signalling. Suppression of SA responses correlated with enhanced JA signalling, showing that HaRxL44 is able to affect the balance between JA and SA signalling, thus enhancing susceptibility to biotrophic *Hpa* (Caillaud *et al.*, 2013).

Other effectors directly targeting JA signalling are HopZ1a and HopX1 from *Pseudomonas syringae*. Both effectors are able to interact with JAZ proteins, which are transcriptional repressors of JA-responsive genes and major components of the JA receptor complex (Jiang *et al.*, 2013; Gimenez-Ibanez *et al.*, 2014). HopZ1a and HopX1 use distinct catalytic activities to induce JAZ degradation. HopZ1a acetyltransferase activity induced JAZ protein degradation in a coronatine-insensitive1 (COI1)–ubiquitin proteasome manner (Jiang *et al.*, 2013), whereas the cysteine protease activity of HopX1 led to JAZ protein degradation independently of COI1 (Gimenez-Ibanez and Solano, 2013). In both cases, effector-mediated degradation of JAZ proteins results in activation of JA signalling and plant susceptibility. Remarkably, some *P. syringae* strains are able to manipulate hormonal homeostasis by producing coronatine (COR), a mimic of bioactive JA-Ile (Fonseca *et al.*, 2009). COR contributes to disease by facilitating bacterial entry through stomatal opening (Melotto *et al.*, 2008) and promoting bacterial multiplication through inhibition of SA signalling by activation of the antagonistic JA pathway (Cui *et al.*, 2005; Laurie-Berry *et al.*, 2006). Interestingly, both HopZ1a and HopX1 partially rescued the virulence defect of COR-deficient *P. syringae* strains, showing that these effectors contribute to bacterial virulence by mimicking COR-induced susceptibility (Gimenez-Ibanez and Solano, 2013; Jiang *et al.*, 2013).

Finally, manipulation of host ABA homeostasis by microbes has been also demonstrated. Indeed, several fungi are able to produce ABA directly (Dörffling *et al.*,1984; Inomata *et al.*, 2004). In other cases, bacterial effectors have been shown to induce host ABA biosynthesis to attenuate

defence responses. For example, AvrPtoB from *P. syringae* and AvrXccC from *X. campestris* are able to overcome plant basal defence by inducing the expression of the ABA biosynthesis *NCED* genes, which disturbs ABA homeostasis through enhanced ABA accumulation (Ho *et al.*, 2013).

Microbial effectors directly target chloroplasts to promote pathogenesis

The importance of chloroplasts as integrators of disease and defence responses is being increasingly highlighted (Stael *et al.*, 2015) and, in addition to microbial strategies to subvert accumulation and signalling of pro-defence molecules discussed earlier, the chloroplast is also emerging as a major direct target for different pathogen effectors (Fig. 1).

As mentioned earlier, PAMP perception results in rapid suppression of NECGs, leading to ROS production and restricting bacterial proliferation (de Torres Zabala *et al.*, 2015; Lewis *et al.*, 2015). The combined action of effectors from *Pst* DC3000 prevents this chloroplastic ROS burst by inhibiting photosynthetic CO_2 assimilation through disruption of PSII (de Torres Zabala *et al.*, 2015). Reprogramming of NECG expression and inhibition of photosynthetic CO_2 assimilation and ROS production are necessary events for bacterial multiplication. Moreover, the effectors HopO1-2 and HopR1 are imported into chloroplasts, showing that *P. syringae* acts both transcriptionally and post-transcriptionally to target the chloroplast. Overall, this work underlines the role of PSII in restricting bacteria and reinforces the importance of the chloroplast in integrating photosynthesis and defence signals (de Torres Zabala *et al.*, 2015).

The first report of pathogen effectors targeting the chloroplast derived from a screen from the type III secretome of *P. syringae* (Guttman *et al.*, 2002). *Pseudomonas syringae* injects ~30 different effectors into host plant cells using the type III secretion system (T3SS), which is essential for pathogenicity of *P. syringae* (Mudgett, 2005). Mutations in a single effector gene generally reduce, but do not impair, the pathogen's ability to cause disease in the host, reflecting that effectors act collectively to defeat plant defences (Kvitko *et al.*, 2009). An example of a collaborative effector is HopI1 from *P. syringae* pv. *maculicola* strain *Pma*ES4326, as *Pma*ES4326 lacking HopI1 only shows attenuated growth in *Arabidopsis thaliana* (Jelenska *et al.*, 2007). HopI1 is a virulence factor that localizes to the chloroplast stroma using a non-canonical import mechanism. Once in the chloroplasts, HopI1 targets the heat shock protein Hsp70, suppressing SA accumulation and chloroplast-mediated SA-dependent responses, altering thylakoid structure and thus reducing host capacity for effective defence (Jelenska *et al.*, 2007, 2010). HopI1 possesses a J domain that is essential for virulence, thylakoid remodelling, and interaction with Hsp70. HopI1 physically interacts with Hsp70, acting as a co-chaperone and increasing the ATP hydrolysis activity of Hsp70. In addition, HopI1 promotes accumulation of cytoplasmic Hsp70 and its recruitment to the chloroplasts, potentially affecting the folding/complex assembly of chloroplast-resident proteins required for SA biosynthesis or transport (Jelenska *et al.*, 2010).

HopN1 is a *Pst* effector able to suppress cell death, ROS production, and callose deposition in *A. thaliana*. Suppression of these immune responses is dependent on HopN1 cysteine protease activity (López Solanilla *et al.*, 2004; Rodríguez Herva *et al.*, 2012). HopN1 was found to interact with the tomato protein PsbQ, one of the members of the oxygen-evolving complex (OEC) of PSII (Enami *et al.*, 2008), previously associated with stress conditions in higher plants (Coker *et al.*, 2005; Ifuku *et al.*, 2005; Gong and Yuan, 2006). HopN1 physically co-localizes with PsbQ in thylakoids and reduces the activity of PSII, probably due to PsbQ proteolysis by HopN1 (Rodríguez Herva *et al.*, 2012). Surprisingly, whilst preventing host cell death by alteration of chloroplastic ROS production, HopN1 did not contribute to bacterial growth *in planta*, supporting the co-operative role of the repertoire of the pathogen effectors in weakening host defences and promoting pathogen proliferation.

An additional *Pst* effector, HopK1, is able to suppress ROS production and callose deposition in plants challenged with the MAMP flg22. The N-terminal part of HopK1 is homologous to the transit peptide of the AvrRps4 effector from *P. syringae* pv. *pisi* and acts as a chloroplast transit peptide. Both effectors are processed *in planta* and localize in the chloroplasts. Expression *in planta* of HopK1 lacking the transit peptide suppressed neither PTI nor ETI, indicating that the targets of this effector, although as yet unknown, may be located in the chloroplasts. Similarly, transit peptide-lacking AvrRsp4 lost its ability to suppress PTI, but retained its HR-inducing activity, probably because AvrRps4 cell death-related function does not require its localization to the chloroplasts (Li *et al.*, 2014). HopK1 and AvrRps4 target(s) remain(s) to be discovered, but it has been proposed that these effectors could target components of the retrograde signalling, thus explaining their contribution to the suppression of both early and late defence responses (Li *et al.*, 2014).

The effector WtsE determines *Pantoea stewartii* ssp. *stewartii* (*Pnss*) virulence and proliferation in maize (*Zea mays*) plants (Asselin *et al.*, 2015). This effector is able to elicit water-soaked disease symptoms in host plants and to promote bacterial growth and survival (Bogdanove *et al.*, 1998; Badel *et al.*, 2006; Ham *et al.*, 2009). WtsE elicits major disruptions in several chloroplast-resident pathways: it suppresses the expression of genes involved in photosynthesis and selectively induces genes involved in shikimate and phenylpropanoid metabolism (Asselin *et al.*, 2015). Chemical disruption of the shikimate pathway suppresses WtsE-mediated pathogenicity. Up-regulation of both the shikimate and the phenylpropanoid pathways elevates the production of phenolic and phenylpropanoid compounds, such as SA and coumaroyl tyramine (CouTyr). Although the shikimate pathway results in numerous metabolites known to promote plant defence, misregulation of phenylpropanoid metabolism could represent a virulence strategy to promote or divert carbon flow into products beneficial or detrimental, respectively, to bacterial survival (Asselin *et al.*, 2015).

In recent work, Petre and co-workers investigated the subcellular localization of 20 candidate effectors of the leaf rust fungus *Melampsora larici-populina* (Petre *et al.*, 2015*b*). One of these secreted proteins was found to be located in the chloroplast when transiently expressed in *N. benthamiana*. It is noteworthy that subcellular localization studies of transiently expressed (candidate) effectors in *N. benthamiana* needs careful examination (e.g. AvrRps4 was localized to the cytoplasm and nucleus when transiently expressed in *N. benthamiana* and to the chloroplast in stable transgenic Arabidopsis lines (Li *et al.*, 2014)). Therefore, it cannot be ruled out that the chloroplast is the target of additional leaf rust effectors. It is still not known how filamentous pathogens enter host cells. In a follow-up work, it was shown that the leaf rust fungus chloroplast-localized effector, named CPT1, carries a transit peptide that is cleaved after translocation into the chloroplast (Petre *et al.*, 2015a). This effector exemplifies pathogen evolution to functionally mimic host-targeting sequences in order to target chloroplasts.

Conclusions

Photosynthesis and immunity are two fundamental processes essential to the life of plants. Although these two processes have been historically investigated in a separate manner, it has become increasingly clear that they are closely connected at several levels. Considering the obvious links between yield, plant health, and photosynthetic efficiency, integrated approaches that investigate the crosstalk between photosynthesis and immunity should provide effective knowledge to be applied for plant protection.

The chloroplast plays a central role in energy production, redox homeostasis, and retrograde signalling to the nucleus that, we now know, collectively contribute to the outcome of the plant immune response. Plant cells rely on the integrated production and delivery of defence-related signals and molecules through different organelles to mount an efficient response to pathogen attack. In addition, organellar production of pro-defence molecules determines gene expression changes in the nucleus, and vice versa. Gaining knowledge on the organization and regulation of this interorganellar crosstalk during plant immune responses is an important task for future research.

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