

On plant defense signaling networks and early land plant evolution

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ABSTRACT

All land plants must cope with phytopathogens. Algae face pathogens, too, and it is reasonable to assume that some of the strategies for dealing with pathogens evolved prior to the origin of embryophytes – plant terrestrialization simply changed the nature of the plant-pathogen interactions. Here we highlight that many potential components of the angiosperm defense toolkit are i) found in streptophyte algae and non-flowering embryophytes and ii) might be used in non-flowering plant defense as inferred from published experimental data. Nonetheless, the common signaling networks governing these defense responses appear to have become more intricate during embryophyte evolution. This includes the evolution of the antagonistic signaling pathways of jasmonic and salicylic acid, multiple independent expansions of resistance genes, and the evolution of resistance gene-regulating microRNAs. Future comparative studies will illuminate which modules of the streptophyte defense signaling network constitute the core and which constitute lineage- and/or environment-specific (peripheral) signaling circuits.

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Introduction

Macroscopic algae and plants are bathed in micro-organisms. Whatever their natural habitat, they are forced to interact with their microbial companions in some manner. Such interactions are diverse in nature. For example, various algae are known to depend on vitamin B₁₂ provided by bacteria in their environment [1]. Another famous example is the “regulation” of algal blooms of the haptophyte *Emiliania huxleyi* by bacteria [2]. Interactions with microbes – both positive and negative – are thus part of every photosynthetic eukaryote’s life. This article will focus on the evolution of the framework that underlies molecular phytopathology in modern-day plants and algae. We review what is known about the recurrent evolution of plant defense signaling networks across streptophyte evolution (Figure 1). In so doing, we span the trajectory from streptophyte algae (the closest extant relatives to land plants [3-5]), mosses, gymnosperms, and angiosperms. Since most data have been gathered for angiosperms, we will use them primarily for comparative purposes.

Evolutionary phytopathology: The nuts-and-bolts of plant-microbe interactions

Common themes in the evolution of plant defense signaling networks become apparent when diverse species from different lineages are compared. Across angiosperm lineages, plant defense signaling is based on core sets of phytohormones (e.g., jasmonic acid; JA) and proteins (e.g., receptors that sense microbial proteins, such as Flagellin sensitive 2; FLS2). Genetic diversity is further shaped by co-evolution driven by arms race dynamics between plants and microbes – affecting, for example, both resistance genes [6,7] and the factors that regulate them, e.g. miRNAs [8,9]. Studying these factors in an evolutionary context has been summarized as the “coming of age” for the study of evolutionary molecular plant-microbe interactions (coined EvoMPMI) by Upson and colleagues [10]. Upson and colleagues [10] emphasized the need for evolutionarily informed studies that focus on a broad scale covering entire land plant diversity as well as on fine-scale variation within or between closely related species.

While the vast body of literature on how land plants deal with phytopathogens is focused primarily on

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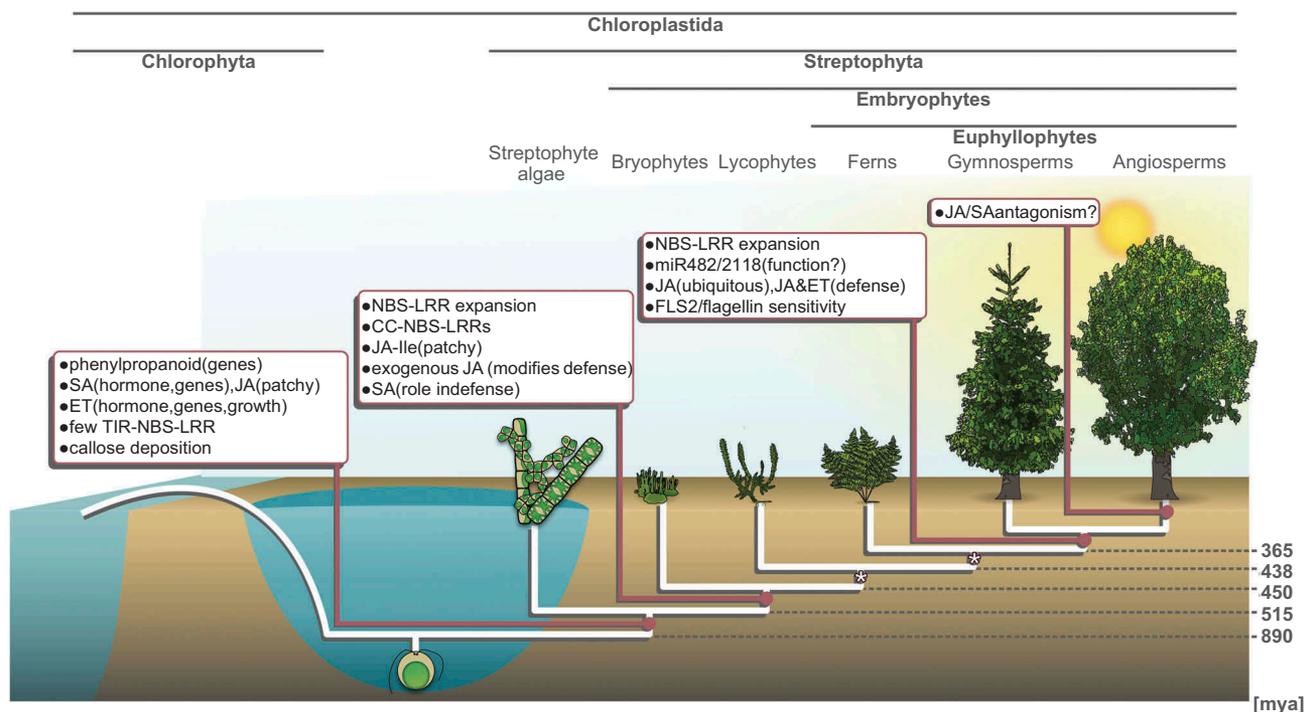


Figure 1. Key phytopathogen interaction factors across the trajectory of streptophyte evolution. Schematic cladogram (white lines) of the Chloroplastida depicts the deep split of the green lineage into Chlorophyta and Streptophyta about 900 million years ago (divergence times based on Morris et al. [45]). The Streptophyta encompass the paraphyletic streptophyte algae and the monophyletic land plants (Embryophyta). Land plants are likely >500 million years old and consist of the non-vascular bryophytes and the ~430 million year old clade of vascular plants, encompassing lycophytes and euphyllophytes. The euphyllophytes are the clade of ferns, gymnosperms and angiosperms (the latter two are the seed plants). Boxes highlight when – along this trajectory – signaling factors in plant defense are thought to have evolved; brackets further specify the type of data and/or functional significance of a given factor. The asterisks indicate nodes for which data are limited. JA, jasmonic acid; JA-Ile, jasmonic acid-isoleucine; SA, salicylic acid; ET, ethylene; NBS-LRR, nucleotide binding site-leucine-rich repeat; TIR-NBS-LRR, Toll-interleukin 1 receptor-nucleotide binding site-leucine-rich repeat; CC-NBS-LRR, coiled coil-nucleotide binding site-leucine-rich repeat; FLS2, FLAGELLIN SENSITIVE2.

angiosperms, research on gymnosperms and bryophytes is catching up [11–14] – yet, as highlighted by Upson et al. [10], ferns and lycophytes have yet to follow suit. Further, at the present time, little is known about the interactions between streptophyte algae and their phytopathogens. As outlined above, understanding commonalities in streptophyte algae and non-flowering plants is important to pinpoint how the defense signaling networks of plants arose. Because of the dynamics in plant-pathogen interactions, however, a plethora of different strategies in plant defense have come about.

Therefore, plant defense mechanisms are composed of common defense strategies as well as lineage-specific ones. A straightforward example of a lineage-specific defense strategy in gymnosperms is the flow of resin in wounded conifers, which depends upon resin ducts. Some resin ducts are formed during plant growth and flooded with resin in response to stress, while other resin ducts are only induced upon infection and wounding through the action of phytohormones [15–17]. By exploring the commonalities and differences, we will highlight both the evolutionary trajectories and

underlying principles of land plant signaling upon phytopathogen attack – including the potential for this signaling in streptophyte algae. We will first consider an example from basal-branching embryophytes and their interactions with substrate-dwelling fungi.

Fungal symbioses exemplify ancient plant-microbe interactions

Symbioses with Glomeromycota-like fungi are hypothesized to have occurred during an early phase of land plant terrestrialization and to have contributed significantly to the global colonization of land [[5,18–20], see also [21]]. Motivated not least by these observations, there is a growing body of literature on bryophyte–fungus interactions.

Various fungal interactions have been observed in liverworts. For example, a recent study by Nelson and colleagues [22] describes several growth-promoting endophytes associated with the liverwort *Marchantia polymorpha*, providing fertile ground for future Evo-MPMI research (see [23]). Other *Marchantia* species

were shown to engage in mutualistic interactions with Glomeromycota [24,25]. And other liverwort genera, such as *Cephalozia bicuspidata* [26], have also been shown to engage in mutualistic interactions with mycorrhizal fungi.

Several bryophytes form mycorrhizae by interacting with fungi [24,27,28], but the picture for bryophytes as a whole is patchy [29]. While many liverworts (outlined above) and hornworts [30,31] exhibit interactions with mycorrhizal fungi, mosses generally do not form mycorrhizae [32,33]; for a recent and comprehensive overview see [29]. That mosses do not form mycorrhizae is further corroborated by Wang and colleagues [34], who showed that moss arbuscular mycorrhizal symbiosis genes show high sequence divergence as compared to their homologous counterparts in all other land plants. Yet, in light of the recently supported monophyly of the bryophytes [35], the phenomenon that mosses do not form mycorrhizae likely represents a case of secondary loss [29]. On balance, symbiosis with arbuscular mycorrhizal fungi appears to be an ancestral feature of all land plants [36]. Indeed, molecular data presented by Wang et al. [34] indicate that genes associated with interactions with mycorrhizal fungi were likely present in the last common ancestor of land plants, which was corroborated by Delaux et al. [18]. But what about the algal progenitors of land plants?

Ancient land plant-microbe interactions and evidence from molecular data in streptophyte algae

Streptophyte algae are known to associate with various kinds of microorganisms. Knack and colleagues [37] performed a metagenomic study of aquatic streptophyte alga- and liverwort-associated microbes, including epiphytic microorganisms (e.g. those growing in the mucilage of streptophyte algae) as well as those colonizing the tissue. Their analyses of three higher-branching streptophyte algae (*Coleochaete pulvinata*, *Chaetosphaeridium globosum* and *Nitella tenuissima*) identified potentially beneficial microbes, for example nitrogen-fixing or cobalamin-producing bacteria, but also potentially harmful ones, such as bacteria associated with cellulose degradation [37]. Interestingly, Knack and colleagues [37] also detected some fungi in metagenomic data, an observation that warrants further investigation. Among the streptophyte algae investigated, the detected signals included sequences stemming from fungi belonging to the Cryptomycota and Chytridiomycota [37]. For the investigated liverwort *Conocephalum conicum* an association with glomalean fungi was demonstrated [37].

As mentioned earlier, Glomeromycota-like fungi feature in discussions revolving around the beneficial symbioses that the earliest land plants engaged in [19,20]. Delaux et al. [18] found that streptophyte algae have most of the genes that land plants put to use during symbiosis signaling. These authors also performed functional complementation experiments in which the capacity to engage in symbiosis with arbuscular mycorrhizal fungi was rescued by heterologously expressing streptophyte algal *CCaMK* in *Medicago ccamk* mutants (which are deficient in interacting with mycorrhizal fungi). This underscores the functional conservation of symbiosis signaling across long evolutionary timescales.

The fossil record also provides insight into streptophyte-fungal symbioses. 400-plus million-year-old *Horneophyton* land plant fossils have been shown to harbor glomeromycotean- and mucoromycotean-resembling structures [38]. Together with the aforementioned molecular data, this information makes a strong case for the idea that the interaction with mycorrhizal fungi is ancient. The genes underlying these beneficial interactions likely predate the origin of the terrestrial flora.

Microorganisms are not only beneficial to plants and algae – they can exploit their hosts as (facultative) phytopathogens [39]. For example, several bacterial and fungal genera or species complexes include mutualistic, pathogenic and endophytic species [39]. Such microbe-host relationships can in fact switch between neutral, beneficial and detrimental in response to, for example, environmental factors [40,41]. It is noteworthy that some of the components necessary for a successful arbuscular mycorrhizal symbiosis, and which are present in streptophyte algae or bryophytes, can also be used for defense signaling [42]. For example, mutations in several symbiosis-associated LysM-RLKs (Lysin Motif Receptor-like Kinases) have been reported to impair defense signaling [42]. In contrast, in the case of *Arabidopsis thaliana*, which does not engage in symbioses with arbuscular mycorrhizal fungi, the oomycete pathogen *Hyaloperonospora arabidopsidis* seems to require components of the arbuscular mycorrhizal symbiosis-associated molecular machinery to successfully complete its life cycle [43]. Hence, “symbiosis genes” might not only tell a tale of ancient mutualism, but also ancient interactions with phytopathogens.

The terrestrial habitat was teeming with microbes before the dawn of land plants [reviewed by [44]]. Hence, during terrestrialization >500 million years ago [see [45] for the latest dating] one can imagine that the earliest land plants would have encountered a very different set of microbes than those in the freshwater environments from which they were emerging.

Yet, a fluent passage scenario seems equally reasonable if one considers, e.g., a freshwater environment (microbe load A) that routinely dried out (microbe load B). It should further be noted that microbe load A and B may also have overlapped given that, for example, many oomycetes and fungi grow equally well in liquid or on solid medium in the laboratory – it goes without saying that this is a mere proxy for what might happen in nature and will require further studies. No matter the scenario, fossils have interesting stories to tell in this case, too.

Taylor et al. [46] reported the existence of a parasitic fungus in a likely more than 400-million-year-old fossil of *Paleonitella*, which appears to be related to extant charophyceae streptophyte algae (such as *Nitella*). But the algal progenitors of land plants would have encountered (terrestrial and/or non-terrestrial) microbes even before this time. Berbee and colleagues [47] recently argued that the occurrence of pectinases (enzymes used for the degradation of pectin in plant cell walls) in even the earliest-diverging fungi [see [48]] argues for the antiquity of the fungal ability to exploit plant material. How so? Pectin is a cell wall component characteristic of land plants and streptophyte algae (reviewed in [49]). Berbee et al. [47] argue that since pectinase-harboring fungal lineages are older than the land plant clade, these fungi used their pectinases for the degradation of streptophyte algal cell walls. This is corroborated by the fact that i) phytoplankton are readily attacked by chytrid fungi [50] and ii) chytrid fungi have been found associated with streptophyte algal microbiomes [37].

In summary, land plants and their closest relatives are, and always have been, associated with both symbiotic and pathogenic microorganisms – their interactions with microbes are truly ancient. Because it is important for hosts to be able to distinguish between a pathogen or a symbiont – and to react accordingly – defense signaling mechanisms must presumably also be present in the algae that are most closely related to land plants. The question that remains is: how similar are these mechanisms in plants and algae? The answer will shed light on the plant-microbe interaction tool kit that was present in the earliest land plants.

PTI and ETI in non-flowering land plants and maybe streptophyte algae

Most of what we know about the plant immune system derives from studying angiosperms. The pathogen recognition system is based upon two components: pattern triggered immunity (PTI) and effector triggered immunity (ETI) [6]. The latter is more specific towards the infecting pathogen because plant resistance genes

(*R* genes) recognize effector proteins secreted by, and specific to, a certain pathogen [51]. PTI causes, for example, stomata closure and cell wall reinforcements at the site of pathogen attack (e.g., through callose deposition, formation of papillae [deposits consisting of callose, phenolic compounds and polysaccharides], and lignification) [52–55]. PTI can also result in cell death caused by the release of reactive oxygen species (ROS) [56]. Additionally, ROS production and thus the initiation of the hypersensitive response (HR) is a classical hallmark of *R* gene-based immunity [51].

Not surprisingly, the potential for PTI can be found in early-branching land plant lineages as well as streptophyte algae (for a comprehensive discussion of genetic potential in streptophyte algae, see [5]). Streptophyte algae such as *Coleochaete* and *Nitella* have been found to contain lignin-like components [49,57–59], potentially used for cell wall reinforcement during pathogen attack. Moreover, the basal-branching streptophyte algae *Klebsormidium* spp. deposit callose in response to abiotic (desiccation) stress [60]. It is further noteworthy that even though Herburger and Holzinger [60] found that *Zygnema* spp. did not deposit callose in response to desiccation stress, callose was nonetheless present in these species. In the moss *Physcomitrella patens*, papillae formation is readily observed close to unsuccessful infection attempts by different *Phytophthora* pathogens [61]. Oomycete and fungal pathogens also induce ROS [62,63] and inoculations with oomycetes resulted in the accumulation of toxic phenolic compounds in *P. patens* [61,62]. Similarly, other mosses, including *Funaria hygrometrica*, also form papillae around fungal penetration sites to prohibit their entry [64,65]. Callose deposition was also observed in the interaction between the liverwort *M. polymorpha* and the oomycete *Phytophthora palmivora* [66].

PTI responses require receptors. One of the best-explored PTI-associated pattern recognition receptors (PRRs) in angiosperms is FLS2. FLS2 recognizes the microbe-associated molecular pattern (MAMP) flg22, a peptide component of the bacterial flagellin [56,67]. Orthologs of FLS2 were not found in the moss *P. patens* [68], although a homolog with appreciable sequence conservation was found [69]. Yet, *P. patens* is flg22-insensitive [70]. Likewise, the receptor for the bacterial translation elongation factor Tu (Ef-Tu), EFR [71,72], seems to be missing outside of the Brassicaceae [68,71] and Ef-Tu does not induce a PTI-like response in *P. patens* [70]. However, the moss does recognize bacteria and mounts a defense response accordingly [73]. This suggests that either more ancient or lineage-specific receptors are used in *P. patens* to recognize bacteria, and that FLS2 and EFR are more recent acquisitions. Indeed, in support of the

presence of a more ancient type of receptor in mosses, *P. patens* is known to respond to bacterial peptidoglycan, which is recognized by the ortholog of the *A. thaliana* receptor CERK1 [70]. Moreover, CERK1 of *P. patens* recognizes chitin from fungi and triggers downstream signaling responses [70]. This might hint that CERK1 was present and functioning in peptidoglycan recognition in the last common ancestor of land plants – but this needs further clarification by investigating CERK1 function across a broader diversity of land plants. In contrast to *P. patens*, protoplasts of the conifer *Pinus thunbergii* produce ROS in response to flagellin treatment [74] and FLS2 is hypothesized to be present in gymnosperms [75]. This suggests that a diversification of PTI-associated PRRs occurred during the evolution of land plants, perhaps associated with the refinement of MAMP-triggered responses.

Components of the heterotrimeric G-protein complex, a signaling switch that consists of an α -, β - and several γ -subunits [76], are involved in land plant defense responses (e.g. [77]); the role of β - and γ -subunits in defense is also implicated to be mediated by FLS2, EF-Tu and CERK1 in *A. thaliana* [78]. Homologs of all three subunits are present in land plants and streptophyte algae [79,80]. Moreover, in the interaction of *P. abies* with the fungal pathogens *Heterobasidion annosum*, *Heterobasidion parviporum* and the saprotroph *Phlebiopsis gigantea*, genes for several subunits of the heterotrimeric G-protein complex were shown to be up-regulated [81]. It was further suggested that this response may be triggered by conserved molecular patterns of the fungi [81], hence possibly associated with PTI. Whether heterotrimeric G-proteins also play a role in defense responses of earlier-diverging land plants and streptophyte algae remains to be investigated.

ETI requires the presence of R proteins to detect pathogen secreted effector proteins either through direct binding or by monitoring whether other host proteins are altered by the actions of effectors [51]; such alterations can include changes in protein conformation and/or phosphorylation status [82,83]. Once R proteins detect an effector protein of a pathogen, they induce pathogen-specific immune responses [84–86]. Nucleotide-binding site leucine-rich repeats (NBS-LRRs) are one of the major classes of R proteins [87]. They are combined with various N-terminal domains, for example the coiled-coil (CC-NBS-LRR) or Toll-interleukin 1 receptor domain (TIR-NBS-LRR) [87].

Potential NBS-LRR-encoding genes have been found from streptophyte algae to angiosperms, but there is pronounced variation in the number of *NBS-LRR* genes

present in any given genome. Conifers have undergone a dramatic expansion of their suite of NBS-LRR genes: while 69 putative *NBS-LRR* genes are predicted for *P. patens* and 16 for the lycophyte *Selaginella moellendorffii*, *P. abies* and *Pinus taeda* have been predicted to possess 562 and 677 putative *NBS-LRR* genes, respectively [88,89]. It is noteworthy that gymnosperms tend to have large genomes (often more than 10 Gbp in size [90]), which could suggest that the expansion of *NBS-LRRs* in plants is related to genome size of the respective plant. Yet, the large genomes of gymnosperms appear to be the result of an expansion of intron size because of repeated insertion of transposable elements and the total number of genes is in fact similar to that observed in *A. thaliana* [90]. Additionally, numbers of *NBS-LRRs* reported in Zhang et al. [88] seem to not necessarily be related to genome size. For example *Medicago truncatula* has “only” a 370 Mbp genome [91], but a similar number of *NBS-LRRs* as *P. abies* [88]. Likewise, the monocot *Triticum aestivum* has a 17 Gbp genome, similar in size to some gymnosperms [92], but has roughly double the number of *NBS-LRRs* than *P. abies* [88].

Species-specific expansions and reductions of *NBS-LRRs* have been observed throughout the Embryophyta [88] – including lineages with differentially expanded *NBS-LRR* subsets. For example, TIR-NBS-LRR-encoding genes are absent from the grasses (Poaceae; [e.g. [88]]). *NBS-LRR* genes also appear to be encoded in the genome of streptophyte algae, but whether they are required for streptophyte algal immunity is currently not known. Yue et al. [69] found three NBS-encoding sequences within the Coleochaetales (higher-branching streptophyte algae), two with sequence similarity to TIR-NBS-LRRs from angiosperms. Furthermore, Urbach and Ausubel ([93; see supplementary appendix) reported the detection of two *TIR-NBS-LRR* genes in the genome of the early-branching streptophyte alga *Klebsormidium nitens* (whose whole genome sequence was reported by [94]). In agreement with this, Gao and colleagues [89] reported three *TIR-NBS-LRRs* in *K. nitens* as well as one *NBS-LRR* with an additional N-terminal domain (*non-TIR-NBS-LRRs*). Several *non-TIR-NBS-LRRs* were found in transcriptomes of six streptophyte algae [89]. Yet, CC-NBS-LRRs (a class of non-TIR-NBS-LRRs) have thus far only been found among land plants, including the moss *P. patens* [88]. It hence appears that one of the most prominent NBS-LRR combinations – the CC-NBS-LRRs – evolved on land.

Given that the recognition of effector proteins by NBS-LRRs results in the initiation of plant cell death, tight regulatory control is essential. Indeed, these proteins are regulated in many ways, including multiple posttranslational mechanisms, such as ubiquitination

and oligomerization with different partners [95]. At the level of expression, they can be regulated by transcriptional as well as post-transcriptional means [95]. The latter is mediated by microRNAs (miRNAs) in angiosperms [96-99]. Several *NBS-LRR*-targeting miRNA families exist, but due to the broad distribution of the miR482/2118 family, this family has received more attention than others.

Members of the miR482/2118 family show low sequence conservation even between closely related species [8]. The family first emerged in gymnosperms [96,100], which seems to coincide with an expansion of *NBS-LRRs* during this time period [88]. The coniferous plant *P. abies* has one of the largest expansions of miR482/2118 [8,100] and the genes likely originated through inverted duplication of *NBS-LRR* genes [100]. miR482/2118 is a direct regulator of resistance to a diverse range of pathogens in dicots [9,98,101-103]. In monocots miR482/2118 is expressed in reproductive tissue and may function in its development [104]. Given the expression patterns of miR482/2118 in *P. abies*, with some members of this family solely expressed in reproductive organs [100], a broader function in the regulation of both reproductive organ development and disease resistance seems to be the more ancient mechanism.

Evolution of phytohormone defense networks

The plant immune system and phytohormone signaling are interwoven [105]. While almost all major phytohormones have been linked to plant immunity at some level [106], jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are key regulators [105]. In angiosperms, SA and JA act primarily antagonistically [107]. SA triggers immunity towards biotrophic pathogens, i.e., those requiring a living host [107]. SA regulates ROS levels by induction as well as scavenging [108]. Furthermore, SA is involved in the induction of HR, resulting in plant cell death [109]. On the other hand, JA is produced in response to herbivores, which induce wounding [110]. In concert with ET, JA also regulates responses towards several necrotrophic pathogens, i.e. those pathogens that actively induce host cell death [107].

It is likely that defense networks similar to those in land plants exist in streptophyte algae. A series of recent studies have revealed the presence of homologs of plant hormone biosynthesis and signaling pathway genes and/or the presence of various phytohormones in streptophyte algae [e.g. [94,111-114]]; yet we are only beginning to understand the function of these phytohormones in streptophyte algae [115-117]. All three

canonical plant defense phytohormones, JA, SA and ET, have been detected in at least some species of streptophyte algae [94,112,115,118]. They also have been explored with regard to pathogen defense in non-flowering land plants. SA has been measured in mosses and gymnosperms – indeed, as in angiosperms, SA has been shown to accumulate in response to elicitors or pathogen attack [63,119,120], supporting its function in defense across land plant diversity.

In streptophyte algae, Ju et al. [112] detected Isochorismate Synthase 1 (ICS1) homologs; ICS1 catalyzes the first step in SA biosynthesis. Furthermore, an ortholog of phenylalanine ammonia lyase (PAL), the enzyme catalyzing the first step in the phenylpropanoid (PP) pathway, was detected in the genome of *K. nitens* [121]. The PP pathway is also a source for SA biosynthesis [122]. Moreover, potential homologs for the SA receptor Nonexpressor of PR genes 1 (NPR1) [123], were reported for all land plants [113] and the putative *NPR1* homolog of *P. patens* can partially complement defense signaling-associated phenotypes of the *Arabidopsis npr1* mutant [124]. As for ET, recent studies showed that streptophyte algae produce, sense and respond to ET [112,115], but these studies did not dissect the role of ET as a hormone involved in defense.

The existence and distribution of JA in early-diverging land plants and streptophyte algae is complex. The canonical pathway genes for JA biosynthesis (13-LOX, 13-Lipoxygenase; AOS, 13-Allene Oxide Synthase; AOC, Allene Oxide Cyclase, OPR3, OPDA Reductase 3 and JAR1, Jasmonate Resistant 1) are present in all land plant lineages [125], and some of its components were also detected in several streptophyte algae [94,112,125]. However, actual (mainly mass spectrometry-based) measurements of JA levels are suggestive of a patchier distribution among land plants and streptophyte algae. For example, while JA is reported to be produced in small quantities in the streptophyte algae *K. nitens* [94] and *Chara australis* [118], Hackenberg and Pandey [126] did not detect JA in *Chara braunii*. Furthermore, Gachet et al. [127] did not detect JA in *Chara vulgaris* and *Klebsormidium elegans*, while Koeduka et al. [128] found only minimal levels of JA in *Klebsormidium flaccidum*. Thus, within the genera *Chara* and *Klebsormidium*, the detection of JA is variable.

Like in *K. flaccidum* (a streptophyte alga) only non-existent or only minimal amounts of JA and its active derivative JA-Ile were detected for *M. polymorpha* (a liverwort) [128,129]. Furthermore, tissue wounding did not increase their amounts [128]. In bryophytes, like in streptophyte algae, JA seems to be produced in a species-specific manner [127,130]. However, JA appears to

be absent from the model moss *P. patens* [131]. Yet, when the moss *P. patens* was infected with two species of *Pythium*, an increase in the production of endogenous JA was detected over time and compared to control plants [62] – although the levels of JA were minimal both before and after infection. In contrast, exposure to the fungal pathogen *Botrytis cinerea* did not result in an increase in JA, but instead an increase in SA and the JA-precursor 12-oxo-phytodienoic acid (OPDA [63]); OPDA also increased after wounding in *M. polymorpha* [129]. These patterns suggest that in bryophytes the function of JA in defense may in fact be conferred by OPDA. Indeed, the signaling pathway of JA in angiosperms is fully functional in *M. polymorpha* [132]. Yet, in contrast to *Arabidopsis*, a derivative of OPDA, 2,3-dinor-OPDA (dn-OPDA), is the functional ligand of the JA receptor ortholog in *M. polymorpha*, Coronatine insensitive 1 (COI1) [132].

Unlike *P. patens*, the model lycophyte *S. moellendorffii* produces JA and is able to sense the phytohormone [133]. However, other lycophytes including another species from the genus *Selaginella* did not produce measurable levels of JA [127], supporting the notion of a high species-specificity in JA biosynthesis. Similarly, some species of ferns show pronounced JA responses, while others do not [134–136]; likewise, the production of JA was shown to be species-specific in ferns [127]. This distribution of JA biosynthesis becomes less patchy in gymnosperms and angiosperms, as shown in the dataset by Gachet and colleagues [127], where only one species in each of these two lineages was identified that did not produce a detectable amount of JA.

How do non-flowering land plants mount their defense responses? In *P. patens*, infection by oomycete and fungal pathogens leads to up-regulation of the usual suspects of angiosperm defense signaling: *PAL*, *Dirigent (DIR)*, *Chalcone synthase (CHS)* and *Pathogen related (PR)* genes, as well as genes involved in JA and JA-precursor biosynthesis, such as *LOX*, *AOS* and *OPR* [62,63,137]. This is, however, not surprising, since infections with *B. cinerea* or two *Pythium* pathogens lead to OPDA production [62,63]. In the spruce *P. abies*, the pathogens *H. parviporum* and *H. annosum* induce the expression of, among other genes, the JA biosynthesis and signaling genes *LOX* and *Jasmonate Zim Domain (JAZ)*, as well as genes for the biosynthesis of ET (*ACO*, *1-aminocyclopropane-1-carboxylic acid [ACC]-oxidase*; *ACS*, *ACC-synthase*), and *PAL*, *DIR2/32* and *PR1* [138–140]. JA and ET act in concert to induce defense responses against necrotrophic pathogens in angiosperms [107]. Hence, the activation of both JA and ET biosynthesis genes in response to

necrotrophic fungal pathogens in *P. abies* suggests a similar interaction between the two phytohormones. In agreement with this, in the two conifers *Pseudotsuga menziesii* and *Sequoiadendron giganteum* the application of MeJA and wounding induce ET biosynthesis, as measured by the activation of *ACO* [16]. In that study, ET was (at least partially) required for the plants' defense responses induced by MeJA and wounding [16]. This suggests that both mosses and gymnosperms not only induce similar defense pathways during infection with necrotrophic pathogens, but also that non-flowering land plants produce immune reactions similar to those observed in angiosperms.

Despite the apparent similarities in immune responses in non-flowering land plants and angiosperms, some differences have been discovered. As mentioned earlier, in the moss *P. patens*, the necrotrophic pathogen *B. cinerea* induced SA production in addition to the biosynthesis of the JA-precursor OPDA [63]. In agreement with this, expression of moss *PpPAL* is induced by SA, JA, MeJA, and OPDA [62,63], suggesting that exogenous JA and SA at least partially activate similar pathways. Indeed, Thaler et al. [141] and Han [125] suggested that the JA/SA-antagonism arose at the earliest in seed plants. Along these lines, it was hypothesized that in the fern *Azolla* some JA-orchestrated signaling responses may be initiated via SA instead of JA because MeSA application induced the expression of *Plant Defensin 1.4 (AfPDF1.4)* [136]; in *Arabidopsis*, *PDFs* are JA-responsive [142]. These results, together with the data from mosses, speak in favor of a reduced antagonism – or perhaps complete lack thereof – between JA and SA in mosses and ferns. In contrast to the hypothesis of Thaler et al. [141], a lack of a canonical antagonism between JA and SA was also suggested for *P. abies* [143]. Both MeJA and MeSA induce marker genes of SA signaling (*PR1* and *Late up-regulated in response to Hyaloperonospora parasitica 1 [LURP1]*) [143]. These genes are also up-regulated in response to the fungal pathogen *H. parviporum*, and upon inhibition of JA signaling, *PR1* expression is significantly reduced after fungal attack [143]. Furthermore, Kozłowski et al. [119] showed that exogenous MeJA can increase SA levels in *P. abies*. In *Ginkgo biloba* an elicitor from *Phytophthora boehmeriae* causes an increase in both endogenous JA and SA [120]. Moreover, both JA and SA were required to produce a defense-associated metabolite in response to the elicitor treatment in *G. biloba* [120]. Yet this study also found that artificially reduced SA led to an increase in JA levels, complementing the loss of SA-derived production of the defense metabolite. This

points to some negative regulatory effects of SA on JA, although the downstream signaling pathways of both hormones do not seem to be antagonistic.

Overall, it seems that JA synthesis was either lost or highly reduced several times throughout the evolution of land plants. Therefore, the requirement for JA in defense responses may be lineage specific. JA precursors, on the other hand, such as OPDA and other oxylipins, are involved in immune signaling in early-branching land plants [144]. As the production of JA became more consistent in gymnosperms and angiosperms, and levels of JA increased compared to earlier-branching lineages, its use in defense signaling was cemented. Long before that, however, at the base of the vascular plants, COI1 acquired a mutation leading to a broader binding pocket, which enabled binding to JA-Ile, the active JA-derivative [132]. After the establishment of JA as another regulator of defense responses, JA and SA signaling evolved into a highly specific antagonistic network.

There are, however, many complexities with regard to the antagonism of JA and SA in *A. thaliana* [145]. Liu et al. [145] showed that SA promotes the synthesis of JA and the activation of its signaling during ETI. However, a recent study by Betsuyaku et al. [146] showed that SA and JA act antagonistically during ETI on a narrow spatial scale. So far, spatial information on JA responses in non-flowering plants is only available for conifers, where MeJA treatment results in cell type-specific PAL activation [17]. Moreover, cell type-specific transcriptomes of *Picea glauca* showed strong cell-specific modulation of gene expression by MeJA treatment, including PP pathway-associated genes, such as PAL [147]. Nevertheless, these studies did not dissect the JA/SA antagonism on spatial scales. Moreover, we cannot exclude the possibility that JA/SA antagonism (or in organisms lacking JA, dn-OPDA/SA antagonism) is lineage-specific in non-angiosperms. However, for the time being, the evidence points to the evolution of JA/SA antagonism with regard to the regulation of defense responses after the split of gymnosperms and angiosperms.

Phenylpropanoids and their derivatives in streptophyte defense responses

Many of the defense- and JA/SA-regulated genes described above encode enzymes in the PP pathway or those downstream of it. PPs and PP-derived compounds, such as lignins, lignans, flavonoids and stilbenes, are defense metabolites, because they i) can be toxic for pathogens and/or ii) reinforce cell wall structures, thereby reducing the possibility of penetration by pathogens [122,148]. PAL encodes

the first enzyme in the PP pathway [122]. It shows a strong responsiveness to pathogens or exogenously applied JA in gymnosperms and JA and SA in mosses [62,63,73,143,149,150]. Therefore, it is not surprising that the defense response of *P. patens* following the inoculation with oomycete and fungal pathogens includes the production of phenolic compounds [61-63]. Cell wall reinforcements in *P. patens* by lignification after *B. cinerea* infection was also suggested because of the enhanced expression of the *Dirigent-like* gene, *PpDIR* [63]; DIR and DIR-like enzymes function both in lignan and lignin formation [151]. Moreover, in a study focused on gene expression of nearly all enzymes required for lignin production in *P. abies*, Koutaniemi and colleagues [138] found that PAL and at least one representative of the nine tested gene families were up-regulated in response to *H. annosum* – a pathogen inducing JA biosynthesis and signaling genes in its host [139]. This points to enhanced lignification as a pathogen defense response in conifers. Indeed, enhanced lignification in cell walls was observed for conifer species from the Cupressaceae and Podocarpaceae after MeJA application [17]. Furthermore, in conifers from different families, the application of MeJA increased the amount of PAL in polyphenolic and ray parenchyma cells [17]. These cell types also accumulated phenolic compounds after the treatment with MeJA in several of the species tested [17].

While it was previously thought that the PP pathway was limited to land plants, de Vries and colleagues [121] showed that streptophyte algae likely possess genes (orthologous to their respective, well-characterized land plant counterparts) for the production of PPs and lignins. As discussed above, a PAL-encoding orthologous gene was detected in the genome of *K. nitens* [121], suggesting that this early-branching streptophyte alga is capable of producing PPs. This is in agreement with the aforementioned detection of lignin-like compounds in streptophyte algae [see 49, 57, 58, 59], which are also derived from the PP pathway. While this suggests that both mechanisms are ancient, we do not know whether PPs and their derivatives are used by streptophyte algae for pathogen and parasite defense.

The expression of flavonoid-associated genes is also triggered by pathogens: Pinaceae up-regulate genes from the flavonoid biosynthesis pathway during infection [149,152]. The expression of flavonoid biosynthesis genes was also correlated with an increase in the flavonoid (+)-catechin in *P. abies* 15 days after infection with *H. annosum* [149]. However, in this study, the increase was genotype dependent, with more susceptible genotypes showing no increase or less of the flavonoid. In *P. patens* flavonoids seem to also play a role in defense responses, as bacterial elicitors as well as oomycete and fungal pathogens induce *CHS* [61,62,73]. Furthermore, other genes of

the flavonoid biosynthesis pathway are induced by bacterial elicitors [153]. In streptophyte algae, several homologs, but few orthologs of the genes required for flavonoid biosynthesis were detected [121]. That being said, Goiris et al. [154] reported the presence of flavonoids in algae from various lineages, including chlorophytes. A 1969 study by Markham and Porter [155] reported on the presence of flavonoids in the charophyceae *Nitella*, highlighting the need to further investigate streptophyte algae with regard to the presence of these metabolites. It is noteworthy that Van de Poel and colleagues [115] found ET-dependent regulation of a homolog of *TRANSPARENT TESTA 8 (TT8)* in the Zygnematophyceae *Spirogyra pratensis*; TT8 is a known regulator of flavonoid biosynthesis [156]. A TT8 ortholog is also present in the dataset for the Coleochaetophyceae *Coleochaete scutata* [114], where it is induced by high light stress.

Conclusion

Angiosperms have evolved complex and fine-tuned regulatory networks to mount their defense responses against microbial pathogens. Many molecular components of these networks can be found in the closest relatives of land plants, the streptophyte algae. We are, however, just beginning to understand whether these pathways are required for streptophyte algal defense responses – and hence likely to have served this purpose in the ancestor of land plants – or whether other pathways are more important in these lineages. We know that non-flowering land plants induce many of these pathways for defense against bacteria, fungi and oomycetes. Defense responses in non-flowering land plants utilize different regulatory modes than do angiosperms, as exemplified by the lack of the JA/SA antagonism in non-flowering land plants (Figure 1). Moreover, regulatory circuits have become seemingly more elaborate throughout land plant evolution, with the expansion of PTI-associated PRRs and NBS-LRRs and the occurrence of NBS-LRR-regulating miRNAs (Figure 1). In conclusion, it seems that many defense pathways of angiosperms existed in the last common land plant ancestor. The same pathways have, however, been reinvented and interwoven during subsequent land plant evolution, resulting in highly intertwined, specific and complex regulatory networks for plant defense.

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