Short title: Host-induced germination of root parasitic plants

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The mechanism of host-induced germination in root parasitic plants

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One-sentence summary:

A family of receptors that evolved in the Orobanchaceae family enable seeds of parasitic plants to sense strigolactones from a nearby host root and germinate.

Author Contributions

DCN wrote the manuscript.

Abstract

Chemical signals known as strigolactones were discovered more than 50 years ago as host-derived germination stimulants of parasitic plants in the Orobanchaceae. Strigolactone-responsive germination is an essential adaptation of obligate parasites in this family, which depend upon a host for survival. Several species of obligate parasites, including witchweeds (*Striga*, *Alectra* spp.) and broomrapes (*Orobanche*, *Phelipanche* spp.), are highly destructive agricultural weeds that pose a significant threat to global food security. Understanding how parasites sense strigolactones and other host-derived stimulants will catalyze the development of innovative chemical and biological control methods. This review synthesizes the recent discoveries of strigolactone receptors in parasitic Orobanchaceae, their signaling mechanism, and key steps in their evolution.

Strigolactones, multifaceted signals in plants and soil

The seed of many parasitic species in the Orobanchaceae can lie dormant for years or decades in soil until chemical signals from a nearby host root activate their germination. This remarkable adaptation is critical for obligate parasites such as witchweeds (*Striga*, *Alectra* spp.) and broomrapes (*Orobanche*, *Phelipanche* spp.), whose survival depends upon attaching to a host soon after germination. Witchweeds and broomrapes are major constraints to crop production and food security in sub-Saharan Africa, Asia, and the Mediterranean region (Xie et al., 2010). The need to develop chemical or biological solutions for this multibillion-dollar agricultural problem has driven a quest to understand how host-triggered germination occurs. Several questions are prominent: 1) What host-derived cues are detected by parasite seeds? 2) How are those cues perceived and translated into a germination response? and 3) How did this adaptation evolve and continue to evolve?

Answers to the first question began in 1966 with the discovery of strigol, a potent germination stimulant of *Striga lutea* that was isolated from cotton (*Gossypium*) root exudates (Cook et al., 1966). In the following decades, more than 20 other parasite germination stimulants with similar chemical structures to strigol have been identified from root exudates (Yoneyama et al., 2018b; Bouwmeester et al., 2020). These molecules compose the family of strigolactones (SLs). Key structural features of SLs and their major classifications are discussed in Box 1.

The structure and stereochemistry of SLs often influences their activity as parasite germination stimulants. For example, *Striga gesnerioides* selectively germinates in response to orobanchol-type SLs but its germination is inhibited by strigol-type SLs (Nomura et al., 2013). Many synthetic SL analogs have been developed during the search for inexpensive, stable compounds that can trigger suicidal germination of parasites in the absence of host. One such analog, GR24, is commonly used to study SL signaling and SL roles in plant development. However, GR24 is frequently used as a racemic mixture (*rac*-GR24) of a 2'*R* configured molecule known as $GR24^{5DS}$ or (+)-GR24, and its unnaturally configured 2'*S* enantiomer, $GR24^{ent\text{-}5DS}$ or (-)-GR24 (Figure 1). These molecules activate two different pathways (Scaffidi et al., 2014; Flematti et al., 2016). In *Arabidopsis thaliana*, $GR24^{5DS}$ signals through D14, while $GR24^{ent\text{-}5DS}$ signals predominantly through KAI2.

Nearly 40 years after the isolation of strigol, an explanation emerged for why plants exude SLs. SLs promote hyphal branching, metabolic activity, and hyphopodium formation of arbuscular

mycorrhizal (AM) fungi, enhancing the ability of the fungi to colonize roots (Akiyama et al., 2005; Besserer et al., 2008; Gomez-Roldan et al., 2008; Kobae et al., 2018). Plants supply AM fungi with carbon in exchange for mineral nutrients. When inorganic phosphate or nitrogen availability is low, symbiosis with AM fungi is particularly beneficial and SL production increases (Yoneyama et al., 2007; López-Ráez et al., 2008; Umehara et al., 2008). A few years after the discovery that SLs affect growth of AM fungi, it was found that SLs are not just signals to the rhizosphere but are also plant hormones that regulate the outgrowth potential of axillary buds, or tillers (Gomez-Roldan et al., 2008; Umehara et al., 2008). Diverse roles for SLs in plant development have since been identified through studies of SL biosynthesis and signaling mutants and application of SL analogs. In addition to shoot branching, SLs regulate stem elongation, auxin transport, root elongation, leaf shape and angle, leaf senescence, secondary growth of the cambium, defense against pathogens and nematodes, stomatal closure, and drought tolerance (Gomez-Roldan et al., 2008; Umehara et al., 2008; Agusti et al., 2011; Kapulnik et al., 2011; Ruyter-Spira et al., 2011; Scaffidi et al., 2013; Shinohara et al., 2013; Bu et al., 2014; Van Ha et al., 2014; Yamada et al., 2014; Lauressergues et al., 2015; Soundappan et al., 2015; Ueda and Kusaba, 2015; Bennett et al., 2016; Lahari et al., 2019; Nasir et al., 2019; Kalliola et al., 2020; Li et al., 2020; Shindo et al., 2020). It may be that non-canonical SLs (see Box 1) function as plant hormones, whereas canonical SLs have external roles primarily (Yoneyama et al., 2018b). In tomato (*Solanum lycopersicum*) at least, loss of both SL types causes obvious developmental phenotypes, but loss of canonical SL production alone does not (Wakabayashi et al., 2019).

Strigolactone perception in non-parasitic angiosperms

To understand how SLs are recognized by the seed of root parasites, it is useful to first discuss how SLs are perceived as hormones by non-parasitic plants. SLs are recognized by DWARF14 (D14)/DECREASED APICAL DOMINANCE2 (DAD2)/RAMOSUS3 (RMS3), which is both an α/β-hydrolase protein and a receptor (Arite et al., 2009; Hamiaux et al., 2012; Waters et al., 2012; de Saint Germain et al., 2016; Yao et al., 2016). SL causes D14 to interact with MORE AXILLARY GROWTH (MAX2)/DWARF3 (D3) (Hamiaux et al., 2012; Zhao et al., 2015; Yao et al., 2016). As an F-box protein, MAX2/D3 confers substrate specificity to an SCF (Skp1, Cullin, F-box) E3 ubiquitin ligase complex. SCF complexes attach polyubiquitin chains to target proteins, which are then rapidly degraded by the 26S proteasome. The targets of D14-SCF^{MAX2} are a subset of proteins in the SUPPRESSOR OF MAX2 1 (SMAX1)-LIKE (SMXL) family that are known as DWARF53 (D53) in rice (*Oryza sativa*) and petunia (*Petunia hybrida*), or SMXL6,

SMXL7, and SMXL8 in *Arabidopsis thaliana.* SL activates association of D14 with these targets. Without D14, MAX2 is likely to have little or no interaction with SMXLs (Jiang et al., 2013; Zhou et al., 2013; Soundappan et al., 2015; Wang et al., 2015; Liang et al., 2016; Shabek et al., 2018; Lee et al., 2020).

The SL signaling mechanism is analogous to gibberellin, auxin, and jasmonate signaling mechanisms. In each of these pathways, hormone perception triggers SCF-mediated degradation of protein targets that indirectly regulate gene expression through association with transcription factors. The targets of auxin and jasmonate signaling also interact directly or indirectly with transcriptional corepressors in the TOPLESS/TOPLESS-RELATED (TPL/TPR) family. Thus, target degradation triggers downstream transcriptional responses (Blázquez et al., 2020). The prevailing hypothesis for SL signaling is that SMXL proteins function similarly as transcriptional co-repressors. SMXL proteins have a conserved C-terminal EAR motif that mediates interactions with TPL/TPR proteins (Jiang et al., 2013; Soundappan et al., 2015; Wang et al., 2015; Ma et al., 2017). In rice, D53 also interacts with the transcription factor IDEAL PLANT ARCHITECTURE1 (IPA1) to regulate expression of *TEOSINTE BRANCHED1* (*OsTB1*), *D53* itself, and other downstream genes (Lu et al., 2013; Song et al., 2017). A similar mechanism is found in wheat. TaD53 interacts with two homologs of IPA1, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE17 (SPL17) and SPL3, to repress *TB1* expression (Liu et al., 2017). Partnering with SPL transcription factors may be specific to monocots, however (Bennett et al., 2016; Wang et al., 2020a). Other transcription factors that presumably associate with SMXL proteins await discovery. Unexpectedly, the model of SMXL function has recently been extended to include SMXL proteins themselves as transcription factors (Wang et al., 2020a). Arabidopsis SMXL6 can bind DNA directly to regulate its own expression as well as that of *SMXL7* and *SMX8*. In addition, SMXL6 works with unknown partners to regulate expression of *BRANCHED1* (*BRC1*), *TCP DOMAIN PROTEIN 1* (*TCP1*), and *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*), which encode key regulators of downstream growth responses to SL. Of the 401 SL-responsive genes that have been identified in Arabidopsis seedlings, 28 genes are directly bound by SMXL6 (Wang et al., 2020a). Putatively, other SMXL family proteins function as transcription factors too, adding an unusual twist to the typical model of F-box mediated phytohormone signaling mechanisms.

Activation of the strigolactone receptor D14

D14 has a strictly conserved Ser-His-Asp catalytic triad that is a common feature of α/β-hydrolase proteins. D14 hydrolyzes SL slowly, and this activity requires an intact catalytic triad (Hamiaux et al., 2012; Seto et al., 2019). Although the byproducts of SL hydrolysis are not thought to be active, catalytic triad mutants demonstrate that hydrolysis is important for D14 signaling activity, with the notable exception of a D218A substitution (Hamiaux et al., 2012; Seto et al., 2019). During SL hydrolysis the methylbutenolide D-ring is opened and cleaved through nucleophilic attack by the catalytic Ser, and ultimately transferred to the catalytic His residue (de Saint Germain et al., 2016; Yao et al., 2016). A crystal structure of Arabidopsis D14 (AtD14) in complex with rice D3 (OsD3) and Arabidopsis Skp1 (ASK1) led to the proposal that the opened D-ring bridges the Ser and His residues in the activated form of D14, forming a covalently linked intermediate molecule (CLIM) (Yao et al., 2016). However, further analyses of the electron density at this site have challenged this interpretation. An iodide ion has been proposed to explain the electron density present in the pocket of activated D14, but a methylbutenolide-His complex seems to provide an even better fit (Carlsson et al., 2018; Bürger and Chory, 2020a). Covalent modification of the catalytic His residue by the D-ring is supported by tandem mass spectrometry analysis of D14 orthologs from Arabidopsis, pea (*Pisum sativum*), and rice (de Saint Germain et al., 2016; Yao et al., 2016; Yao et al., 2018b). Eventually, the D-ring can be released, enabling a new round of SL hydrolysis at least *in vitro*. However, D14 is degraded within a few hours after SL treatment in Arabidopsis and rice (Chevalier et al., 2014; Hu et al., 2017). If this is a common feature of angiosperms, D14 proteins that have a slow rate of D-ring release, such as RMS3 in pea, are likely to function as “one-shot” enzymes (de Saint Germain et al., 2016).

Pea and rice D14 proteins show biphasic SL hydrolysis activity *in vitro* consisting of a brief “burst” phase of rapid hydrolysis followed by a “plateau” phase of slow hydrolysis (de Saint Germain et al., 2016; Shabek et al., 2018). Putatively, the plateau phase is due to the rate at which the enzyme can discharge the SL byproducts (especially the D-ring) and reset. However, Arabidopsis D14 has not shown a biphasic hydrolysis response to the synthetic SL analog GR24 (Box 1; Seto et al., 2019). It is not clear whether this difference reflects the species origin of the D14 or the ligands used. These observations of D14 enzymatic activity are potentially complicated by the use of fluorogenic reporter molecules and SL analogs whose byproducts after hydrolysis may dissociate at different rates than those of natural SLs. The ratio of enzyme to ligand also affects the ability to observe the biphasic response (Shabek et al., 2018). Finally, the presence of D3 and D53 can influence the *in vitro* rates of SL hydrolysis by D14, potentially

by stabilizing D14 in different conformations (Shabek et al., 2018). Notably, a major conformational change to D14 and a covalently attached SL byproduct have only been observed when D3 was co-crystallized (Yao et al., 2016). It is unknown how association of D14 with SCF^{MAX2} and SMXL proteins influences its reaction kinetics *in vivo*. It is also currently under debate which stage of the process of SL binding and hydrolysis by D14 activates signal transduction (de Saint Germain et al., 2016; Yao et al., 2016; Shabek et al., 2018; Seto et al., 2019). For an excellent critical discussion of the several models for D14 activation that have been proposed, see (Bürger and Chory, 2020a).

The mechanism of karrikin perception

Remarkably, the SL signaling pathway is an evolutionary innovation of a pathway that mediates responses to karrikins (KARs) (see excellent review by Machin et al., 2020). KARs are a class of butenolide molecules found in smoke, biochar, and soil after a fire that act as plant growth regulators (Figure 1) (Flematti et al., 2004; Kochanek et al., 2016; Hrdlička et al., 2019). The capacity to respond to KARs is likely to be widespread among angiosperms, and is not restricted to species from fire-prone ecosystems (Nelson et al., 2012).

KAR signaling is similar to SL signaling in several ways. KAR responses are mediated by SCF^{MAX2} and a D14 paralog known as KARRIKIN INSENSITIVE 2 (KAI2), HYPOSENSITIVE TO LIGHT (HTL), or D14-LIKE (D14L) (Nelson et al., 2011; Sun and Ni, 2011; Waters et al., 2012). SMAX1 and SMXL2, which are paralogs of D53-type SMXL proteins, act downstream of MAX2 and KAI2 (Stanga et al., 2013; Stanga et al., 2016). Upon activation, KAI2 interacts with SCF^{MAX2} and SMAX1 or SMXL2, triggering polyubiquitination and degradation of the SMXL proteins (Figure 2) (Khosla et al., 2020a; Zheng et al., 2020; Wang et al., 2020b). This pathway regulates many processes in plants, including seed germination, hypocotyl or mesocotyl elongation, cotyledon expansion, seedling responses to light, leaf shape, cuticle development, drought tolerance, root skewing, root hair density and elongation, and the capacity for AM fungal symbiosis (Shen et al., 2007; Nelson et al., 2009; Nelson et al., 2010; Sun and Ni, 2011; Stanga et al., 2013; Gutjahr et al., 2015; Soundappan et al., 2015; Stanga et al., 2016; Li et al., 2017; Swarbreck et al., 2019; Villaécija-Aguilar et al., 2019; Carbonnel et al., 2020; Choi et al., 2020; Zheng et al., 2020)

The details of KAI2 activation are less understood than for D14, partly because it remains unclear what ligand(s) KAI2 perceives. Genetic studies clearly show that KAI2 is necessary for

KAR responses (Waters et al., 2012). There is also ample biochemical evidence that KAI2 from several species can bind KAR_1 *in vitro* (Guo et al., 2013; Kagiyama et al., 2013; Toh et al., 2014; Xu et al., 2016; Lee et al., 2018; Bürger et al., 2019). These observations have supported the idea that KAI2 is a KAR receptor. However, other data strongly suggest that KAR_1 requires metabolism by plants to become a ligand for KAI2. First, the affinity of KAI2 for KAR_1 *in vitro* is typically one to two orders of magnitude lower than the biologically effective concentrations. Second, KAI2-KAR_1 crystal structures from two species have not shown a consistent orientation of KAR_1 in the ligand-binding pocket, so it is unclear which, if either, captures a true binding pose (Guo et al., 2013; Xu et al., 2016). Third, in multiple assays *in vitro* and *in vivo*, KAI2 is unresponsive to KARs but is activated by $GR24^{ent\text{-}5DS}$, which has a stereochemical configuration not found in natural SLs (see Box 1; Figure 1; Flematti et al., 2016). Differential scanning fluorimetry (DSF) can detect shifts in the melting temperature (T_m) of a protein in response to a candidate ligand, and has emerged as a very useful tool for studying activation of D14 *in vitro* (Hamiaux et al., 2012; Abe et al., 2014; Hamiaux et al., 2018; Seto et al., 2019; Yasui et al., 2019). In DSF assays of AtKAI2, $GR24^{ent\text{-}5DS}$ but not KAR_1, KAR_2, or $GR24^{5DS}$ triggers a T_m decrease (Waters et al., 2015; Yao et al., 2018a). Corresponding with this putative readout of KAI2 activation, yeast two-hybrid interactions between KAI2 and SMAX1 are stimulated by $GR24^{ent\text{-}5DS}$ but not by KAR_1, KAR_2, or $GR24^{5DS}$ (Khosla et al., 2020a). KAI2 can pull down SMAX1 and SMXL2 expressed in protoplasts in the presence of $GR24^{ent\text{-}5DS}$. However, KAI2 does not pull down SMXL2 in the presence of KAR_1 (Wang et al., 2020b). Similar results are obtained for KAI2-MAX2 interactions. Yeast two-hybrid interactions between KAI2 and MAX2 are weakly enhanced in the presence of *rac*-GR24 (a racemic mixture of $GR24^{5DS}$ and $GR24^{ent\text{-}5DS}$), but not KAR_1. *In vitro* pulldown interactions between KAI2 and MAX2 are stimulated by *rac*-GR24 but not KAR_1 (Xu et al., 2018). Finally, KAR_1 treatments require several-fold longer incubations than $GR24^{ent\text{-}5DS}$ to stimulate polyubiquitination and degradation of SMXL2 protein in Arabidopsis seedlings (Wang et al., 2020b). Therefore, KAR_1 and $GR24^{ent\text{-}5DS}$ are not equivalent agonists of KAI2; while $GR24^{ent\text{-}5DS}$ is "ready-to-go," KAR_1 clearly is not. It is currently hypothesized that the normal function of KAI2 in plants is not transducing KAR signals, but sensing an unknown endogenous signal known as KAI2 ligand (KL) (Nelson et al., 2011; Conn and Nelson, 2015; Waters et al., 2015; Sun et al., 2016). It may be that the hydrolyzable D-ring of $GR24^{ent\text{-}5DS}$ makes it a better substitute for KL than unmetabolized KARs.

Evolution of strigolactone perception in angiosperms

SL biosynthesis has an ancient origin in land plants. The complete set of SL biosynthetic pathway enzymes is found in all major land plant lineages, with the possible exception of hornworts. *Physcomitrium* (formerly *Physcomitrella*) *patens* and *Marchantia polymorpha*, which are used as models of basally diverged land plants, have lost one or more SL biosynthesis genes, but this is not representative of bryophytes. (Walker et al., 2019). Sampling for SLs outside of angiosperms is still somewhat limited, but orobanchol-type SLs have been reported in the lycophyte *Selaginella moellendorffii* and gymnosperms (Yoneyama et al., 2018a; Yoneyama et al., 2018b). The moss *P. patens* is reported to only produce carlactone (the biosynthetic precursor of carlactonoic acid and SLs), and not canonical SLs (Alder et al., 2012; Seto et al., 2014; Yoneyama et al., 2018b). However, an unknown SL-like signal(s) derived from carlactone seems likely. This signal inhibits protonemal growth of moss and can also stimulate germination of the parasitic plant *Phelipanche ramosa* (Proust et al., 2011; Lopez-Obando et al., 2020).

The emergence of SL signaling in land plants is less clear (Walker et al., 2019; Blázquez et al., 2020; Machin et al., 2020). In terms of MAX2-associated receptors, *KAI2* orthologs are found in early diverging land plant lineages, such as *Physcomitrium*, *Selaginella*, and *Marchantia*. Phylogenetic analysis indicates that *D14* arose from an early duplication of *KAI2*, but clear *D14* orthologs are apparent only in seed-bearing plants (spermatophytes, *i.e.* gymnosperms and angiosperms) (Bythell-Douglas et al., 2017). Similar to *D14*, the proteins targeted by SL signaling emerged during later phases of land plant evolution. *SMXL* genes are found throughout land plants. However, *SMAX1* orthologs emerged in spermatophytes, and *D53* orthologs (e.g. *SMXL6*, *SMXL7*, and *SMXL8*) evolved later, after the angiosperm lineage diverged (Walker et al., 2019). Divergence within the SMXL family may be associated with specialized functions in plant development and the co-evolution of receptor-target pairs (Waters et al., 2017; Blázquez et al., 2020). Notably, SMAX1-type and D53-type SMXL proteins typically have different roles. For example, in Arabidopsis SMAX1 and its paralog SMXL2 regulate germination and hypocotyl elongation, whereas SMXL6, SMXL7, and SMXL8 regulate axillary bud outgrowth (Soundappan et al., 2015; Stanga et al., 2016). However, some developmental processes, such as rice mesocotyl elongation, are regulated by both types of SMXL proteins (Zheng et al., 2020).

Although the genetic components of canonical SL signaling are angiosperm-specific, it is plausible that an analogous SCF^{MAX2}-dependent signaling mechanism mediates SL responses in other land plant clades. However, recent studies provide support for a KAI2-SCF^{MAX2}

signaling system that mediates responses to KL but not necessarily SL in *Marchantia polymorpha* and *Physcomitrium patens*. *Marchantia polymorpha* has a relatively simple set of genes for this pathway, with only two *KAI2*, one *MAX2*, and one *SMXL*. *Mpkai2a* and *Mpmax2* mutants have highly similar phenotypes that are suppressed by loss of *MpSMXL*, as expected from the pathway found in angiosperms, whereas the *Mpkai2b* mutant has no obvious phenotype (Mizuno et al., 2020). High concentrations of *rac*-GR24 affect thalli growth, but in a *MpMAX2*-, *MpKAI2a*-, and *MpKAI2b*-independent manner. Only a 2'*S*-configured GR24 enantiomer, GR24$^{ent\text{-}4DO}$, induces thermal destabilization of the MpKAI2 proteins *in vitro*, supporting that they do not respond to naturally configured SLs (Mizuno et al., 2020). The signaling system is more complex in *Physcomitrium patens*, which has 13 *KAI2-LIKE* (*PpKAI2L*) genes. It was initially proposed that some of the less-conserved PpKAI2L proteins might function as SL receptors in *Physcomitrium patens*, which makes a carotenoid-derived transmissible signal and responds to *rac*-GR24 and GR24^{5DS} (Proust et al., 2011; Hoffmann et al., 2014; Lopez-Obando et al., 2016; Lopez-Obando et al., 2020). However, a biochemical analysis of several PpKAI2L proteins only showed evidence of binding KAR_1 or unnaturally configured 2'*S* SLs (Bürger et al., 2019). Moreover, the *Ppmax2* mutant is phenotypically different from a carlactone-deficient mutant (Lopez-Obando et al., 2018). An extensive genetic analysis of the *PpKAI2L* family now shows that the five members of the PpKAI2L(A-E) clade, which are grouped among the eu-KAI2 proteins that are conserved in all land plants, are likely to control growth in coordination with PpMAX2. On the other hand, proteins in the PpKAI2L(JGM) clade are proposed to mediate responses to carlactone-derived signals in a PpMAX2-independent manner (Lopez-Obando et al., 2020). Studies of additional species outside the angiosperms will be required to determine whether these results are representative.

Distinct germination responses to KARs and SLs in autotrophs and parasites

There are clear differences in the germination responses of *Arabidopsis thaliana* and parasitic Orobanchaceae seed to KAR and SL. Arabidopsis seed germinates in response to both treatments, but *rac*-GR24 is clearly less potent than KARs (Nelson et al., 2009). By contrast, the obligate parasites O*robanche cernua*, *O. crenata*, *O. cumana*, *O. minor*, *Phelipanche aegyptiaca*, *P. ramosa*, or *Striga hermonthica* respond well to *rac*-GR24 but are altogether insensitive to KARs (Fernández-Aparicio et al., 2009; Nelson et al., 2009; Scaffidi et al., 2014; Conn et al., 2015; Brun et al., 2019). If we consider KARs as indicators of low competition (due to fire) and SLs as indicators of nearby plants, these selective germination responses seem

well-suited for autotrophic and auxotrophic plants, respectively. Germination of obligate parasites in response to KARs that appear after fire would likely be suicidal, for example.

Several lines of evidence indicate that SLs do not regulate Arabidopsis germination. Primary dormant seed of the SL-deficient mutants *max1*, *max3*, and *max4* and the SL-insensitive mutant *d14* have normal germination. In contrast, *kai2* and *max2* mutants have clearly enhanced dormancy (Nelson et al., 2011; Waters et al., 2012). Similar germination trends are observed among *max* mutants under light-restricted conditions (Shen et al., 2012). Therefore, Arabidopsis germination is controlled by KAI2-SCF^{MAX2}-dependent signaling, but not by SL signaling through D14. Indeed, the 2'*S* configured molecules $GR24^{ent\text{-}5DS}$ and *ent*-5-deoxystrigol (*ent*-5DS, an enantiomer of the canonical SL 5-deoxystrigol) promote Arabidopsis germination, but other stereoisomers of GR24 and natural SLs do not (Scaffidi et al., 2014). These responses correspond to the stereoselective activation of KAI2 *in vitro* and *in vivo* (Scaffidi et al., 2014; Waters et al., 2015; Khosla et al., 2020a; Wang et al., 2020b).

It might be argued, however, that the exclusive control of germination by KAI2 is only a feature of primary dormant seed. For the commonly used Arabidopsis ecotype Col-0, primary dormancy typically is lost within several days of afterripening and varies between seed batches, making this a difficult trait to study. Imbibition of after-ripened Arabidopsis seed at supraoptimal temperatures imposes thermoinhibition, a form of secondary dormancy (Toh et al., 2012). This approach has become a powerful tool for evaluating *MAX2*-dependent germination responses in Arabidopsis (Toh et al., 2015; Uraguchi et al., 2018; de Saint Germain et al., 2020). SL biosynthesis mutants have been reported to be hypersensitive to seed thermoinhibition, suggesting that SLs contribute to germination under these conditions (Tsuchiya et al., 2010; Toh et al., 2012). However, an Arabidopsis *kai2/htl* line that has acquired extraordinary, picomolar sensitivity to applied SLs through introduction of *ShHTL7*, a SL receptor transgene from *Striga hermonthica* (see below), still does not germinate under thermoinhibited conditions without a SL treatment (Toh et al., 2015). If ShHTL7 can sense the non-canonical SLs produced by Arabidopsis (Abe et al., 2014; Seto et al., 2014; Brewer et al., 2016; Yoneyama et al., 2018a; Yoneyama et al., 2018b), this suggests that there is little or no SL in Arabidopsis seed, even under thermoinhibition conditions. It is not yet clear whether a lack of germination responses to SLs is peculiar to Arabidopsis or a common feature of non-parasitic angiosperms. However, it seems that autotrophic plants would be at a competitive disadvantage when germinating in response to SLs, which are exuded from established plants already starved for nutrients.

Identification of novel SL receptors in parasitic Orobanchaceae

The discoveries that D14 was likely a SL receptor and its homolog KAI2 regulates seed germination set the stage for understanding SL perception in parasitic Orobanchaceae. It was hypothesized that in parasites a *MAX2*-dependent mechanism had been co-opted for SL-responsive germination (Nelson, 2013). This could occur if D14 became a germination regulator, or if KAI2 evolved the ability to recognize SLs. Supporting the first idea, Arabidopsis D14 can crosstalk with SMXL2 when an analog of 4-deoxyorobanchol, $GR24^{4DO}$, is supplied (Wang et al., 2020b). Putatively D14 can also crosstalk with SMAX1, which has a bigger role than SMXL2 in hypocotyl elongation, as *kai2* seedlings respond to *rac*-GR24 treatment but *kai2 d14* seedlings do not (Waters et al., 2012; Scaffidi et al., 2014; Stanga et al., 2016). Because the *d14* mutant does not show phenotypes consistent with SMAX1 or SMXL2 overaccumulation, however, it is unlikely that D14 normally regulates these proteins. Furthermore, expression of *D14* under the control of a *KAI2* promoter does not rescue germination of *kai2*, even with *rac*-GR24 treatment (Conn et al., 2015; Waters et al., 2015). Therefore, to regulate germination, D14 might require changes that enhance its expression in seed as well as its affinity for SMAX1-type SMXL proteins. By contrast, evolution of SL perception in a KAI2 protein would require a switch in stereochemical selectivity to accommodate 2'*R* configured SL molecules as ligands.

An investigation of *D14* and *KAI2* genes in parasitic plant genomes and transcriptomes was carried out, thanks largely to resources developed by the Parasitic Plant Genome Project and 1000 Plants Initiative (Westwood et al., 2012; Conn et al., 2015; One Thousand Plant Transcriptomes Initiative, 2019). This revealed an unexpected, and sometimes dramatic, expansion of *KAI2* copy number in several parasites (Conn et al., 2015). Although *D14* appears to be a single copy gene in non-parasitic and parasitic species in the Lamiids, *KAI2* has undergone extensive duplication in many facultative and obligate parasite genomes (Figure 3). The *Striga asiatica* genome has 21 *KAI2* paralogs, and *Striga hermonthica* is likely to have at least 13 (Conn et al., 2015; Toh et al., 2015; Tsuchiya et al., 2015; Yoshida et al., 2019). Lamiid *KAI2* genes are distributed into three phylogenetic groups undergoing different rates of evolution. Both parasitic and non-parasitic Lamiids have one or two *KAI2* copies that are grouped within a "conserved" clade (*KAI2c*). Many non-parasitic Lamiids and *Striga* spp. have one or two copies of *KAI2* that are grouped within a "intermediate" grade (*KAI2i*) under weaker purifying selection than *KAI2c*. Parasitic Orobanchaceae uniquely carry a third type of rapidly evolving, "divergent" *KAI2* (*KAI2d*) that often comprise the majority of *KAI2* paralogs in a

species (Figure 3) (Conn et al., 2015). Because gene duplication can enable the evolution of different gene functions, KAI2d proteins were attractive candidates for SL receptors.

Indeed, biochemical studies have provided compelling evidence that *KAI2d* genes encode SL receptors. A fluorogenic agonist, Yoshimulactone Green (YLG), was developed to monitor the hydrolytic activity of SL receptors (Tsuchiya et al., 2015). This enabled *in vitro* YLG competition assays, which test the inhibitory effects of a candidate ligand on the rate of YLG hydrolysis by a SL receptor. The half-maximal inhibitory concentration (IC_{50}) value provides an indirect assessment of the affinity a receptor has for a given SL. Of the two KAI2i- and eight KAI2d-class HTL proteins tested from *Striga hermonthica*, ShHTL6 (KAI2d class) and ShHTL7 (KAI2d class) have low IC_{50} (< 500 nM) for several SLs. This likely indicates high affinity, as ShHTL7 has similar K_m and IC_{50} values for strigol of 57 nM and 120 nM, respectively. Other KAI2d-class ShHTL proteins show more selective ligand preferences among five SLs tested in YLG competition assays (Tsuchiya et al., 2015). Isothermal calorimetry assays provide further support that KAI2d proteins have high affinities for SL. Several KAI2d-class ShHTL proteins bind 5-deoxystrigol with K_d values in the ~40 nM to 4 μM range (Wang et al., 2021).

Further evidence that KAI2d proteins are SL receptors comes from cross-species complementation experiments, which provide a convenient way to evaluate the function of an individual *KAI2* gene *in vivo*. *Arabidopsis thaliana* is well-suited for this purpose due to the availability of KAR and SL pathway mutants, easy transformation, and a two-month generation time. In these assays, Arabidopsis *kai2* mutants are transformed with *KAI2* transgenes from parasites and tested for responses to SLs and other germination stimulants. Although some transgenes are not functional - which could reflect a loss of function in their native context, or incompatible interactions with signaling partners in Arabidopsis - many *KAI2d* transgenes from *Striga hermonthica*, *Phelipanche aegyptiaca*, and *Phelipanche ramosa* confer clear germination responses to SLs, *rac*-GR24, and/or 2'*R* configured GR24 stereoisomers (Conn et al., 2015; Toh et al., 2015; Khosla and Nelson, 2016; de Saint Germain et al., 2020). Transgenic Arabidopsis lines carrying *ShHTL7* are striking examples, with germination responses to picomolar concentrations of several SLs and *rac*-GR24 (Toh et al., 2015). Similarly, a fusion protein of GFP and *Phelipanche ramosa* KAI2d3 confers germination responses to ~10 picomolar (+)-GR24 to the Arabidopsis *kai2* mutant (de Saint Germain et al., 2020). PrKAI2d3 shows a clear preference for 2'*R* configured SL analogs, and is several orders of magnitude less responsive to 2'*S* configured analogs.

The ability of a parasite *D14* gene to regulate Arabidopsis seed germination has not been reported. Therefore, while there are no obvious evolutionary signatures, such as gene duplication or evidence of positive selection, to suggest that *D14* may have gained new functions in parasites, it is still formally possible that it could contribute to SL-responsive germination. Although loss-of-function mutations are not available to test the roles of *KAI2* and *D14* genes in parasites directly, chemical tools have provided a way forward. Sphynolactone-7 (SPL7, not to be confused with SPL transcription factors) was developed as a synthetic agonist of *Striga hermonthica* germination (Uraguchi et al., 2018). The potency of SPL7 is very similar to that of the natural SL, 5-deoxystrigol (5DS); both can induce germination effectively at concentrations of ~100 fM. SPL7 is different from 5DS, however, because it shows a high degree of selectivity for ShHTL7 and, to a lesser extent, ShHTL8. *In vitro* YLG competition assays show that SPL7 has an IC_{50} of 0.31 μM for ShHTL7, 1.2 μM for ShHTL8, and 7.8 μM for ShHTL11. By contrast, SPL7 has an IC_{50} >10 μM for D14, the KAI2i-class proteins ShHTL2 and 3, and the KAI2d-class proteins ShHTL4, ShHTL5, ShHTL6, ShHTL9, and ShHTL10 *in vitro* (Uraguchi et al., 2018). Although not all ShHTL proteins have been tested and their relative abundance in *Striga* seed is unknown, this suggests that ShHTL7 is primarily responsible for detecting SPL7. If so, then activation of ShHTL7 is apparently sufficient to trigger *Striga hermonthica* germination. By contrast, ShD14 is not required, at least for responses to SPL7.

Further support for the importance of ShHTL7 in SL-responsive germination is potentially provided by the non-ionic surfactant Triton X-100, which reduces germination responses of *Striga hermonthica* to *rac*-GR24 (Shahul Hameed et al., 2018). Triton X-100 binds to ShHTL7 *in vitro*, blocking *rac*-GR24-induced structural rearrangements of ShHTL7 and its interaction with ShMAX2. Homology modeling suggests that ShHTL7, but not other ShHTL proteins, can accommodate Triton X-100 in their active sites (Shahul Hameed et al., 2018). Therefore, if the inhibition of *Striga hermonthica* germination by Triton X-100 is due to interfering with SL perception and not a nonspecific effect, these data indicate that ShHTL7 is critical for GR24 responses. This does not exclude the possibility that other ShHTL proteins contribute to germination responses to 5DS or other SLs. If selective inhibitors can be developed for other ShHTL proteins, their individual contributions to host-induced germination could be evaluated.

Where strigolactone perception occurs

Based on a detailed anatomical and physiological analysis, perisperm cells adjacent to the micropyle have been proposed to be the site of host-chemical detection in *Phelipanche aegyptiaca* (Joel et al., 2012). In support of this, transcripts of *CYP707A1*, an ABA 8'-hydroxylase that acts downstream of SL signaling, accumulate in these perisperm cells specifically after several hours of GR24 treatment (Lechat et al., 2012).

A fluorogenic chemical probe has also been used to examine the sites and timing of SL perception in *Striga hermonthica* (Tsuchiya et al., 2015). YLGW is a brighter, but less specific, form of YLG. It is hydrolyzed *in vitro* by several ShHTL proteins but not by ShD14, ShHTL7, and a few other ShHTL proteins. After seed coat removal, fluorescence microscopy of *Striga hermonthica* embryos reveals two waves of YLGW hydrolysis that putatively report the activity of SL receptors. Fluorescence is observed in the embryonic root tip within minutes, then diffuses apically during a "wake-up" phase that occurs over several hours. After a pause during which the fluorescence signal dissipates, germination begins. As the root grows, an "elongation tide" of fluorescence emerges in what appears to be the elongation and differentiation zones. These fluorescence patterns were not observed in non-conditioned seeds, which do not respond to SL (Tsuchiya et al., 2015). Because ShHTL7 and ShD14 do not hydrolyze YLGW, this implies that neither is required for the wake-up phase of SL perception. If YLGW hydrolysis is ShHTL dependent, these results imply the enzymatic activity of other ShHTL proteins.

The mechanism of strigolactone perception in parasitic seed

KAI2d proteins in parasites perceive SL in a highly similar manner to D14 (Figure 2). ShHTL7 and ShHTL4 hydrolyze *rac*-GR24 and 5DS, whereas ShHTL1 (KAI2c class) and ShHTL3 (KAI2i class) have little or no hydrolytic activity on these substrates (Xu et al., 2018). As a consequence of SL hydrolysis, the methylbutenolide D-ring becomes attached to the catalytic His residue of KAI2d proteins, as was observed for D14. This was shown through mass spectrometry of ShHTL7 after 5DS and *rac*-GR24 hydrolysis, and PrKAI2d3 in the presence of *rac*-GR24 (Yao et al., 2017; Uraguchi et al., 2018; de Saint Germain et al., 2020). Similar covalent modification of the catalytic His residue in ShHTL7 occurs after treatment with a broad range of SPL7 analogs (Uraguchi et al., 2018). Interestingly, PrKAI2d3 also confers weak responses to the hydroxymethylbutenolide product of SL hydrolysis (D-OH), which is ineffective at activating SL signaling (Hamiaux et al., 2012; de Saint Germain et al., 2020). However, because D-OH is ~10,000 times less potent than *rac*-GR24 at activating *Striga hermonthica* germination, this may not be a biologically significant reaction.

KAI2d proteins very likely function in cooperation with MAX2, as is the case for KAI2 in non-parasitic species. *ShMAX2* is able to rescue many, but not all, mutant phenotypes of Arabidopsis *max2*, demonstrating at least partially conserved functions (Liu et al., 2014). Likewise, the ability of *ShHTL* transgenes to stimulate seed germination or regulate hypocotyl elongation of *Arabidopsis thaliana* is *MAX2*-dependent (Bunsick et al., 2020). Homology modeling of the ShHTL7-ShMAX2 complex shows an interface that is well-conserved with that of AtD14-MAX2 (Shahul Hameed et al., 2018). Many of the amino acids at this interface are also highly conserved in the broader eu-KAI2 protein family in land plants (Bythell-Douglas et al., 2017). Finally, ShHTL4, ShHTL5, ShHTL7, ShHTL8, and ShHTL9 physically interact with ShMAX2 in the presence of *rac*-GR24 and natural SLs, similar to ShD14 (Yao et al., 2017; Shahul Hameed et al., 2018; Xu et al., 2018; Wang et al., 2021).

Interactions between KAI2 and SMAX1 proteins in parasites have not been tested, however, there is indirect evidence that this is likely to occur. First, *KAI2d* transgenes from parasites are able to regulate Arabidopsis germination and seedling growth, which are exclusively or predominantly controlled by SMAX1 among the SMXL family members in Arabidopsis (Stanga et al., 2016). Second, GR24 enhances pull-down interactions between ShHTL7 and Arabidopsis SMAX1, but not Arabidopsis SMXL6 (Yao et al., 2017). This is consistent with the preference that AtKAI2 shows for SMXL partners, and suggests that only the ligand-binding capacity of KAI2d proteins has changed in parasites (Soundappan et al., 2015; Khosla et al., 2020a). Third, ShHTL5, ShHTL7, ShHTL8, and ShHTL9 proteins interact with Arabidopsis SMAX1 in yeast two-hybrid assays in the presence of *rac*-GR24 or natural SLs. When AtMAX2 is coexpressed, ShHTL interactions with SMAX1 are strengthened. Likewise, the C-terminal “D2” domain of Arabidopsis SMAX1 enhances *in vitro* interactions between ShHTL7 and AtMAX2 (Wang et al., 2021).

These observations collectively indicate that KAI2d proteins in parasites have retained the function of KAI2 proteins in stimulating degradation of SMAX1 via SCF^{MAX2}, but are activated by SLs instead of KL or KAR. Thus, *D14* and *KAI2d* have convergently evolved into SL receptors from duplicated *KAI2*.

Downstream effects of KAI2 activation

Although activation of KAI2d proteins can reasonably be expected to cause SMAX1 degradation in parasites, how this leads to germination has been less clear. Recent studies have made progress toward understanding crosstalk between SMAX1 degradation and other hormone pathways that regulate seed germination. Two major players in control of physiological seed dormancy are abscisic acid (ABA) and gibberellic acid (GA), which have antagonistic effects as inhibitors and promoters of germination, respectively. Ethylene also promotes germination of many species, including some parasites, at least in part through inhibition of ABA levels and signaling (Arc et al., 2013). Crosstalk between these hormones is complex.

ABA degradation is an important component of SL-induced germination of *Phelipanche ramosa* seed. Application of *rac*-GR24 quickly and transiently induces expression of *CYP707A1*, which encodes an ABA-catabolizing enzyme. Corresponding with this, ABA levels decline several-fold in conditioned seeds after GR24 treatment. Inhibition of ABA catabolism with the CYP707A inhibitor abscinazole-E2B reversibly blocks GR24-induced germination (Lechat et al., 2012). A similar induction of *CYP707A1* occurs in the obligate parasites *Orobanche cumana*, *O. minor*, and *Striga hermonthica* upon treatment with *rac*-GR24. In contrast, the facultative hemiparasite *Triphysaria versicolor* does not require host-derived stimulants and has only a modest germination response to *rac*-GR24. Induction of *CYP707A1* in *T. versicolor* seed by *rac*-GR24 is similarly low. Thus, SL-induced catabolism of ABA may be more important for germination of obligate parasites than facultative hemiparasites. Indeed, the enhanced seed dormancy of obligate parasites relative to *T. versicolor* may be due to their increased sensitivity to ABA (Brun et al., 2019).

Initial physiological studies of KAR responses in Arabidopsis showed that KAR_1 could not recover germination of GA-deficient mutants. KAR_1 also induces expression of GA biosynthesis genes *GA3ox1* and *GA3ox2* in seed, while not affecting GA sensitivity, suggesting that GA is required for KARs to stimulate Arabidopsis germination (Nelson et al., 2009). However, when activated with *rac*-GR24, several KAI2d-class ShHTL proteins expressed in Arabidopsis are able to override the GA requirement (Bunsick et al., 2020). A similar effect is achieved through a *smax1* loss-of-function mutation, which can overcome germination inhibition by the GA biosynthesis inhibitor paclobutrazol. The differences between these studies might be due to the degree to which SMAX1 is removed from Arabidopsis seeds; *i.e.* KAR_1 activation of AtKAI2 may not be as effective at reducing SMAX1 protein levels as *rac*-GR24 activation of ShHTL or a *smax1* mutation. Regardless, experiments with *Striga hermonthica* support the idea that SL

signaling can activate germination independently of GA in parasitic plants. GA had little effect on promoting *Striga hermonthica* germination, while GR24 stimulated *Striga* germination even in the presence of paclobutrazol (Bunsick et al., 2020).

It is possible that KAI2d proteins stimulate germination in parasites by inducing production of ethylene. In contrast to paclobutrazol, the ethylene biosynthesis inhibitor aminoethoxyvinylglycine is effective at blocking GR24-stimulated germination of *Striga hermonthica* (Tsuchiya et al., 2015). In Arabidopsis, SL signaling through D14 triggers dark-induced leaf senescence in a feed-forward loop with ethylene signaling (Ueda and Kusaba, 2015). This invites speculation that KAI2-regulated SMXL proteins might also regulate growth, at least partially, through ethylene. Notably, under light-grown conditions ethylene can overcome a GA-deficiency to stimulate Arabidopsis germination (Karssen et al., 1989). Furthermore, regulation of root and root hair development in *Lotus japonicus* and Arabidopsis by KAR/KL is due to upregulation of ethylene biosynthesis after degradation of SMAX1 (Carbonnel et al., 2020).

How KAI2 diversification in parasites may affect host range

During the antagonistic coevolution of parasitic Orobanchaceae and their host plants, an increased capacity for SL perception is likely to have been valuable for a parasite. This could enable the parasite to expand its host range, counterbalance evolution of alternative SLs in hosts, or prevent an evolutionary dead-end when a host species becomes extinct locally. For obligate parasites of crops (*i.e.* weeds), whose populations can change dramatically from year to year, a broadened SL response could be a key adaptation. For example, this likely enabled the recent expansion in host range for a new race of *Orobanche cumana* (Dor et al., 2020). Expansion of the *KAI2d* gene family in parasitic Orobanchaceae potentially enables detection of a greater variety of SLs. Indeed, although the dataset is limited, there seems to be a trend of more *KAI2d* paralogs in the genomes of weedy parasites, which can attack many crop species, compared to species with specialized parasitic relationships (Figure 3; Conn et al., 2015). At the other end of the spectrum, one *KAI2c* gene but no *KAI2d* gene was able to be identified in a *de novo* transcriptome assembly for *Conopholis americana,* which parasitizes oak and beech trees (Figure 3; Conn et al., 2015). Perhaps this parasite does not require a mechanism for its seed to detect the presence of a host because its hosts are so long-lived.

571 Because so much emphasis has been placed on studying parasitic weeds, however, it is
572 important to remember that many parasitic Orobanchaceae are not generalists, but instead
573 have a narrow host range. From the point of view of an obligate parasite, an inherent danger of
574 SL-responsive germination is that not all SL-exuding plants are compatible hosts. Plants mount
575 an array of barriers against parasitism, ranging from physical fortifications to immune responses
576 (Clarke et al., 2019). Specialization for different hosts can be observed even among races of a
577 parasitic species, and likely drives speciation (Thorogood et al., 2009). As germination
578 responses are one component of host-specialization, it is not surprising that obligate parasites
579 can show strong germination response preferences for root exudates from specific species
580 (Fernández-Aparicio et al., 2009; Fernández-Aparicio et al., 2011b). Presumably, this is due to
581 the presence of specific SLs in some exudates but not others, as obligate parasites show
582 specific germination responses to different strigolactones (Fernández-Aparicio et al., 2011b;
583 Nomura et al., 2013).
584
585 Putatively, some KAI2d proteins have evolved the ability to detect or prefer specific SL(s).
586 Alternatively, some KAI2d proteins may have become highly sensitive to a broad range of
587 ligands. Both cases have been observed among ShHTL proteins through YLG competition
588 assays and cross-species complementation experiments (Toh et al., 2015; Tsuchiya et al.,
589 2015). It should be noted, however, that the SL preferences of KAI2d proteins *in vitro* do not
590 always reflect which SLs are effective stimulants of seed germination. For example, ShHTL6
591 and ShHTL10 have a very low IC_{50} for orobanchol (58 nM and 390 nM, respectively), yet *Striga*
592 *hermonthica* seed are relatively unresponsive to orobanchol (Tsuchiya et al., 2015). Thus, the
593 emergent germination response to host-derived stimuli may depend upon the relative
594 abundance and activities of the suite of available KAI2d proteins. Although parasite KAI2d
595 proteins have so far been considered activators of germination, I hypothesize that the function
596 of some KAI2d proteins could be to *inhibit* germination after perception of a specific non-host SL
597 (see Box 2). A combination of positive and negative responses to different SLs could produce
598 finely tuned host-specific germination responses.
599
600 Several non-canonical SLs and lactone molecules that can stimulate germination of parasitic
601 Orobanchaceae have been identified (reviewed in Yoneyama et al., 2018b; Bouwmeester et al.,
602 2020). These include zealactone, avenaol, heliolactone, dehydrocostus lactone, and
603 peagoldione (Evidente et al., 2009; Joel et al., 2011; Kim et al., 2014; Ueno et al., 2014;
604 Charnikhova et al., 2017). It is plausible that these molecules are also perceived by KAI2d

proteins, but their specific receptors await discovery. Notably, dehydrocostus lactone induces *CYP707A1* expression in seed of the sunflower (*Helianthus annuus*) parasite *Orobanche cumana*, similar to *rac*-GR24, implying it is perceived through a similar system (Brun et al., 2019). Like KARs, dehydrocostus lactone lacks a cleavable D-ring; it may be that it also requires metabolism before perception by *Orobanche cumana*.

KAI2d agonists are not necessarily restricted to lactones. *Phelipanche ramosa* is a widespread parasitic weed that has recently expanded its host range to include oilseed rape (*Brassica napus*). *Phelipanche ramosa* seed germinate in response to isothiocyanates derived from glucosinolate breakdown in *Brassica napus* root exudates, albeit with about four orders of magnitude less sensitivity than to SLs (Auger et al., 2012). Triton X-100 and KK094 inhibit the ability of GR24 to induce *Striga hermonthica* germination, and at least partially interfere with SL perception by ShHTL7 (Shahul Hameed et al., 2018; Nakamura et al., 2019). Addition of these antagonists blocks the germination-stimulating effect of 2-phenethyl isothiocyanate (2-PEITC), suggesting that 2-PEITC may signal through KAI2d protein(s) (de Saint Germain et al., 2020). PrKAI2d3 has recently been reported to undergo a T_m shift in the presence of isothiocyanates, although it is less substantial than the shift induced by GR24. Remarkably, 2-PEITC becomes covalently bound to the catalytic Ser of PrKAI2d3, suggesting a mechanism for activating the receptor (de Saint Germain et al., 2020). The sensitivity of *Phelipanche ramosa* populations to different germination stimulants varies according to the host they were isolated from. *Phelipanche ramosa* seed sourced from *Brassica napus* fields are uniformly responsive to 2-PEITC, whereas *Phelipanche ramosa* seed sourced from tobacco (*Nicotiana tabacum*) or hemp fields show reduced or heterogeneous responses to 2-PEITC (Huet et al., 2020). Comparisons of *KAI2d* sequences and expression levels among these populations should provide fascinating insights into how rapid shifts in host-stimulant perception can evolve. Assuming dehydrocostus lactone is perceived by a KAI2d protein(s), a similar comparison of *KAI2* evolution in the recently diverged species *Orobanche cumana* and *Orobanche cernua* will also be informative.

This hypothesis awaits investigation. Meanwhile, it is notable that ShHTL10 and ShHTL11 have high affinities for SLs *in vitro* but are inactive when expressed in Arabidopsis (Toh et al., 2015; Tsuchiya et al., 2015; Wang et al., 2021). *ShHTL10* and *ShHTL11* are grouped in a *Striga*-specific *KAI2d* subclade with *ShKAI2d2*, which is also inactive in Arabidopsis (Conn et al.,

2015). These three proteins have substitutions at several well-conserved surface residues that may affect interactions with MAX2 or SMAX1 (Khosla and Nelson, 2016; Wang et al., 2021). Indeed, ShHTL10 and ShHTL11 do not interact with AtMAX2 or ShMAX2 in yeast two-hybrid assays in the presence of SLs (Wang et al., 2021). The activity of these proteins might explain the reduced germination responses of *Striga hermonthica* to orobanchol-type SLs.

Structural basis of ligand-specificity and SL sensitivity in parasitic KAI2d

While there is substantial evidence that KAI2d proteins are responsible for SL perception, very limited surveys suggest that KAI2c and KAI2i proteins in parasitic plants mediate KL and/or KAR perception instead (Conn et al., 2015; Conn and Nelson, 2015; Toh et al., 2015). Therefore, comparisons of the amino acid sequences and three-dimensional structures of parasitic KAI2 are expected to reveal how different ligand-specificities and affinities are achieved within this family. Crystal structures have been solved for ShD14 and several KAI2/HTL proteins from *Striga hermonthica*, including ShHTL1/ShKAI2c, ShHTL3/ShKAI2i, ShHTL4, ShHTL5, ShHTL7, and ShHTL8 (Toh et al., 2015; Xu et al., 2016; Xu et al., 2018; Zhang et al., 2020). This wealth of information, as well as homology modeling and mutational analysis, have revealed several factors that influence ligand recognition: pocket size, ligand-positioning residues, and residues that guard the pocket entrance. In the following discussion of these insights, it is important to remember that some mechanistic details derived from *in vitro* or *in silico* single-protein experiments with KAI2/HTL may differ *in vivo*, where signaling partners or other cellular factors may affect a receptor's structure and activity.

Homology modeling predicts that the ligand-binding pockets of most KAI2d proteins are unusually large compared to KAI2c or KAI2i proteins (Conn et al., 2015; de Saint Germain et al., 2020). Indeed, crystal structures of ShHTL4, ShHTL5, ShHTL7, and ShHTL8 reveal pockets with more than twice the volume of AtKAI2, ShHTL1/ShKAI2c, or ShHTL3/ShKAI2i. These pockets are also larger than those found in D14 proteins from rice and *Striga hermonthica* (Xu et al., 2018; Zhang et al., 2020). Residues at positions Y124, W153, F157, and F194 are among those that influence pocket volume (n.b., residue identifiers are based on AtKAI2). These residues are usually highly conserved in angiosperm KAI2 proteins, but have undergone extensive substitutions to less bulky amino acids in KAI2d proteins (Conn et al., 2015). The substitutions that have occurred at Y124 and S196 in KAI2d proteins are predicted to better accommodate the D-ring of GR24, while non-conservative changes at W153, F194, and A219 are proposed to affect ligand positioning (Toh et al., 2015). The pocket volume is also

673 influenced by shifts in the positioning of the lid helix αD1, which forms part of the pocket
674 entrance (Xu et al., 2018). In ShHTL7, helix αD1 tilts away from the pocket entrance more than
675 in ShHTL/KAI2 proteins that have smaller pockets. The shift of helices αD1 and αD2 away from
676 αD3, which enlarges the pocket, is proposed to be due to an Y150F substitution in the αD1-αD2
677 loop (Xu et al., 2018). Variable substitutions at this position occur in some, but not all, KAI2d
678 proteins in parasites.
679
680 By contrast, the pocket of ShHTL1/ShKAI2c has a smaller volume than AtKAI2, and
681 ShHTL3/ShKAI2iB is smaller yet (Xu et al., 2016; Xu et al., 2018). While these proteins can bind
682 KAR_1 *in vitro*, their pockets are likely to be too small to accommodate SLs without substantial
683 conformational changes upon binding. Indeed, ShHTL1 and ShHTL3 do not hydrolyze *rac*-
684 GR24, and ShHTL1 only has very weak hydrolytic activity against 5DS (Xu et al., 2016; Xu et
685 al., 2018). Likewise, Arabidopsis lines carrying *ShHTL1/ShKAI2c* and *ShHTL3/ShKAI2i*
686 transgenes are not responsive to *rac*-GR24 or SLs, but *ShKAI2i* lines are responsive to KARs
687 (Conn et al., 2015; Conn and Nelson, 2015; Toh et al., 2015). The small pocket of ShHTL1 is a
688 product of bulky residues, for example at position 190 (Xu et al., 2018). The ShHTL3 pocket is
689 influenced more by an inward shift of helix αD1 that reduces the pocket volume and closes the
690 entrance. Conformational shifts have been observed for αD1 in different ShKAI2iB structures,
691 suggesting this helix could act as a gate-keeper for ligand entry and exit (Xu et al., 2016).
692
693 The significance of many binding pocket residues for the ligand affinity of KAI2 proteins has
694 been examined through site-directed mutations. Substitution of residues 124, 190, and 194 in
695 ShHTL7 with bulky amino acids that reduce pocket volume causes a >100-fold decrease in *rac*-
696 GR24 binding affinity *in vitro* (Xu et al., 2018). Position 124 may be particularly important. This
697 residue is highly conserved as Tyr in KAI2c and Phe in KAI2i, but is substituted with smaller
698 hydrophobic amino acids in KAI2d (Conn et al., 2015). Substitutions of the Leu at this position
699 with Phe or Tyr in ShHTL8 caused 10- to 100-fold increases in IC_{50} for *rac*-GR24 in YLG
700 competition assays and disrupted *rac*-GR24 hydrolysis (Zhang et al., 2020). Hydrolysis of *rac*-
701 GR24 was also decreased by I124F substitutions in ShHTL6, but not ShHTL4. Conversely,
702 substitutions of Tyr or Phe with Leu at this position enabled *rac*-GR24 hydrolysis by
703 ShHTL1/ShKAI2c and ShHTL2/ShKAI2i (Zhang et al., 2020). A recent in-depth biochemical
704 analysis of the ShHTL7 binding pocket has revealed substitutions at 18 residues that reduce the
705 binding affinity for *rac*-GR24 more than 10-fold. G25A, M139K, and mutation of the catalytic Ser
706 (S95A) have particularly strong effects, with at least a 200-fold reduction in *rac*-GR24 affinity.

Interestingly, T157Y appears to cause a ~10-fold increase in affinity for *rac*-GR24 *in vitro* (Pang et al., 2020).

A more refined understanding of how ligand specificity is determined in KAI2 proteins is beginning to emerge. Due to potential steric clashes, residues 142, 157, 218, and 219 are thought to contribute to specificity in binding 2'*R versus* 2'*S* stereoisomers of GR24 and KAR_1 (Xu et al., 2018). Residues at positions 96 and 189 are important contributors to KAR_1 vs. KAR_2 selectivity in Brassicaceae KAI2 proteins (Sun et al., 2020). Likewise, amino acid identity at positions 157, 160, and 190 influences the ability of KAI2 paralogs in *Lotus japonicus* to recognize $GR24^{ent\text{-}5DS}$ (Carbonnel et al., 2019). In *Physcomitrium patens* KAI2L proteins, the loop between αD2 and αD3 contributes to ligand specificity, putatively by influencing the formation and rigidity of the ligand-binding pocket (Bürger et al., 2019). A combination of molecular docking and molecular dynamics simulations was used recently to examine interactions between eight SL receptors (including four D14 and four ShHTL proteins) and 20 canonical and non-canonical SLs (Bürger et al., 2020b). This *in silico* approach builds upon the static snapshots of protein structure provided by X-ray crystallography to provide detailed predictions of a protein's conformational dynamics and substrate-binding behaviors "in solution." The analysis suggested that an inflexible internal bottleneck can limit access of some SL molecules to the binding pocket of SL receptors and therefore may be an important component of ligand specificity. By contrast, the outer entrance to the pocket does not appear to be a limiting factor for SL compatibility. The pocket itself has enough volume and flexibility to accommodate internal rotation of SLs to optimal binding poses for SL hydrolysis. Interestingly, molecular dynamics simulations predict that the catalytic Ser and His residues are important for positioning SL in the pocket correctly. Pocket residues at KAI2-equivalent positions 157, 134, 142, 193, and, to a lesser degree, positions 26 and 124 were implicated in frequently forming contacts with docked SLs (Bürger et al., 2020b).

A major question to be addressed is how some SL receptors in parasites, such as ShHTL7 and PrKAI2d3, are able to achieve such extraordinary sensitivity to SLs. It is important to note that ShHTL7 is not particularly remarkable compared to other KAI2d proteins *in vitro*. In YLG competition assays, ShHTL7 has IC_{50} values for various SLs in the ~0.1 to 1 μM range, comparable to ShHTL6 and ShHTL8 (Tsuchiya et al., 2015). Neither does ShHTL7 show an unusually high or low rate of GR24 hydrolysis *in vitro* compared to other KAI2d proteins in *Striga hermonthica* (Tsuchiya et al., 2015). However, in germination assays of transgenic

741 Arabidopsis, the EC_{50} of *rac*-GR24 for ShHTL7 lines is ~20 pM, about 1000 times lower than the
742 ~30-100 nM EC_{50} observed in lines carrying ShHTL6 or ShHTL8 (Toh et al., 2015). ShHTL4 and
743 ShHTL5 also confer higher sensitivity to SL *in vivo* than would be expected from YLG
744 competition assays (Toh et al., 2015; Tsuchiya et al., 2015). Clearly, the affinities that KAI2d
745 proteins show for SLs *in vitro* are not sufficient to account for their germination-promoting
746 activities *in vivo* (Shahul Hameed et al., 2018). Instead, KAI2d proteins that confer particularly
747 sensitive germination responses to SL must be more effective at activating downstream signal
748 transduction. This could occur if a receptor is more readily activated or its activated state more
749 persistent. This is a difficult mystery to resolve, not least because of the current disagreements
750 about what constitutes activation of a SL receptor. While some models for D14 have focused on
751 formation of CLIM (or rather, a covalently modified catalytic His residue) during SL hydrolysis,
752 others propose that SL binding is sufficient for signaling and hydrolysis is a subsequent
753 deactivation step (Yao et al., 2016; Seto et al., 2019). Alternatively, enhanced signal
754 transduction could occur if a receptor has higher affinity for MAX2 and/or SMAX1 upon
755 activation than other KAI2d proteins. New evidence supports this hypothesis (Wang et al.,
756 2021). Among 11 ShHTL proteins tested with *in vitro* pull-down assays, ShHTL7 shows a clearly
757 enhanced ability to interact with AtMAX2 in the presence of SL. Substituting five residues at the
758 MAX2 interface of ShHTL6 with ShHTL7 amino acid identities dramatically increases ShHTL6
759 affinity for AtMAX2 in pull-downs to ShHTL7 levels (Wang et al., 2021). *In vivo* assays of this
760 ShHTL6 mutant are needed to establish whether it can confer germination responses to
761 picomolar SL concentrations, similar to ShHTL7.
762
763 The ShHTL7-specific agonist SPL7 and its analogs show that hydrolysis of the D-ring is
764 dispensable for SL signaling, but comes at a very high cost in potency (Uraguchi et al., 2018).
765 SPL7 and its demethylated analog H-SPL7 have minimum effective concentrations on *Striga*
766 *hermonthica* germination of 10 fM and 10 pM, respectively. By contrast, their hydrolysis-
767 resistant analogs require 6 to 8 orders of magnitude higher concentrations to achieve a similar
768 effect. Thus CLIM formation is important for effective signaling. However, the correlation
769 between the rate of CLIM formation *in vitro* and potency *in vivo* is weak. This is illustrated by a
770 series of SPL7 analogs that separately modify the scaffold and D-ring, or by a direct comparison
771 of SPL7 and GR24, which have identical D-rings. Although CLIM formation in ShHTL7 is about
772 10 times slower with SPL7 than GR24, SPL7 is ~1000 times more potent for *Striga* germination.
773 Therefore, interaction of the ABC-ring of SLs or the scaffold of SL analogs with the pocket of

KAI2d, presumably after hydrolysis, also appears to be important for highly effective signal transduction (Uraguchi et al., 2018).

Long-timescale molecular dynamics simulations have been used to compare SL perception in ShHTL7 and AtD14, and identify potential explanations for their different levels of SL sensitivity (Chen et al., 2020). These *in silico* simulations predict that ShHTL7 is more efficient than AtD14 at binding GR24 in a productive pose that favors hydrolysis. In part this is due to more stable associations of GR24 with hydrophobic residues at the entrance to the D14 pocket that slow its binding. Also, D14 is more prone to dwelling in nonproductive conformations in which the D-loop of D14, which contains the catalytic Asp residue, extends outside the core of the protein. Finally, the volume of the ShHTL7 pocket fluctuates within a narrower range than D14, reducing small-volume conformations that prevent GR24 binding or large-volume conformations that allow non-productive orientations of the ligand (Chen et al., 2020). These exciting hypotheses may be able to explain at least some of the SL hypersensitivity of ShHTL7. Mutations of ShHTL7 have demonstrated which residues are important for maintaining high sensitivity to agonists (Uraguchi et al., 2018; Xu et al., 2018; Pang et al., 2020). However, specific mutations that cause a KAI2 protein to mimic ShHTL7 by increasing its affinity for SL *in vitro* or SL-signaling activity *in vivo* have not been reported. This will be an important goal to demonstrate a true understanding of how highly sensitive SL perception occurs in parasite seed.

Origins and implications of the *KAI2* expansion in Orobanchaceae

The dramatic *KAI2* expansion observed in several parasite genomes is likely due to unequal crossover events, which cause localized *cis*-duplications. These duplications originated before the parasitic lineage diverged, as tandem or near-tandem *KAI2* duplications can be observed in non-parasitic Asterid relatives of the Orobanchaceae. In tomato (*Solanum lycopersicum*), two pairs of tandem copies of *KAI2* are located on chromosome 2. One pair has a *KAI2c* adjacent to a *KAI2i*, and the other pair has a likely pseudogene *KAI2* adjacent to a *KAI2c*. In *Mimulus guttatus*, two *KAI2c* are separated by two genes on the same scaffold. The third *KAI2* paralog, a *KAI2i* is found on another scaffold. The first draft genome sequence for a parasitic plant, *Striga asiatica*, has provided further insights into the genomic distribution of the amplified *KAI2* family. The *Striga asiatica* genome has several examples of functional *KAI2* paralogs and *KAI2* pseudogenes that are linked on the same scaffold (Yoshida et al., 2019). Curiously, duplication of *D14* is relatively uncommon in angiosperms (Conn et al., 2015). It is possible that there is

purifying selection to maintain a single copy of *D14*. Alternatively, *KAI2* may be surrounded by sequences that make it more prone to unequal crossover events. Genome sequences for *Striga hermonthica* and *Orobanche cumana* are expected to be released soon, and will provide useful points of comparison.

Genetic linkage between *KAI2d* paralogs has several implications. First, the capacity to perceive a set of SLs may be heritable as a haplotype block, potentially enabling rapid spread of a beneficial, multigenic trait through a parasitic population. Second, the *KAI2d* family may be able to expand or contract relatively rapidly through additional unequal crossovers. Gene conversion may also influence the diversity of the *KAI2d* repertoire. Third, it will likely be very difficult to connect perception of a specific SL to a single *KAI2d* through recombination-based trait mapping.

Overcoming limitations to genetic studies of *KAI2* function in parasites

KAI2d proteins in parasites have been convincingly implicated in the perception of host-derived germination stimulants through *in vitro* SL-binding and SL-hydrolysis assays, cross-species complementation, and application of KAI2-specific inhibitors. However, genetic analysis in parasitic plants would provide a more conclusive and direct evaluation of the functional contributions of individual *KAI2d* genes. This would be a formidable pursuit through classical genetic methods due to functional redundancy and genetic linkage among parasite *KAI2* families. Reverse genetic approaches, particularly the use of CRISPR-Cas9 gene editing technology, offer a way forward if efficient methods for parasite transformation are available.

Transient and stable transformation of the facultative hemiparasites *Triphysaria versicolor* and *Phtheirospermum japonicum* has been achieved with *Agrobacterium tumefaciens* or *A. rhizogenes* (Tomilov et al., 2007; Ishida et al., 2011; Bandaranayake and Yoder, 2018). A method for stable transformation of the obligate holoparasite *Phelipanche aegyptiaca* has also been developed, producing transgenic roots and shoot buds (Fernández-Aparicio et al., 2011a). These approaches enable evaluation of gene function in parasite roots. However, fertile plants have not been regenerated, so the ability to induce the heritable genetic changes needed for evaluation of germination phenotypes is still lacking.

A breakthrough in transformation of recalcitrant dicots was achieved recently (Maher et al., 2020). Expression of developmental regulators such as *WUSCHEL*, *BABY BOOM*, and *SHOOT*

MERISTEMLESS during *Agrobacterium*-mediated transformation can induce *de novo* formation of meristems from leaf and stem tissues. This method successfully generates heritable transgenic or gene-edited events from plants grown in sterile culture or in soil. Because this approach bypasses the need to regenerate shoots and roots from callus tissue, which is a common roadblock to tissue culture-based transformations, it may be a promising tool for genetic manipulation of parasitic plants.

In terms of prioritizing targets, gene expression patterns may provide clues about which *KAI2* paralogs have major roles in germination control. So far, comparisons of the expression of *KAI2d* paralogs in different parasite tissues are limited to *Striga hermonthica* and *Striga asiatica*. Interestingly, while the transcripts of some *KAI2d* paralogs are enriched in seeds or induced during seed conditioning, others show seedling-specific expression patterns (Tsuchiya et al., 2015; Yoshida et al., 2019). This raises the possibility that there may be a role for host-derived SL perception after germination.

Translational outcomes of understanding the host-detection mechanism

An ongoing goal of the SL field is the discovery of stable and inexpensive chemical substitutes for SLs that can either stimulate suicidal germination of parasitic weeds or block parasite germination in the field. Many approaches to finding parasite germination regulators have been guided initially by assays of hormonal SL activities, such as tillering/shoot branching repression in non-parasitic angiosperms or yeast two-hybrid interactions between D14 and D53 (for example, Nakamura et al., 2019). Others have used assays for activation of Arabidopsis KAI2 (AtKAI2). For example, a yeast two-hybrid assay for AtKAI2 interaction with MAX2 was used to screen a chemical library of 4,182 compounds. Forty-two compounds that promoted AtKAI2-MAX2 interactions in yeast were further screened with seedling growth and CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)-localization assays in Arabidopsis, leading to the identification of three lead compounds. All three compounds promoted germination of *Striga hermonthica*, albeit with much lower potency than GR24 (Toh et al., 2014). A screen for antagonists that interfere with GR24 perception in Arabidopsis seedlings, which occurs through AtKAI2 and D14, was performed with the same chemical library (Holbrook-Smith et al., 2016). Thirty-seven compounds blocked the inhibitory effect of *rac*-GR24 on Arabidopsis hypocotyl elongation, of which seven inhibited germination of Arabidopsis seed. The best performing lead compound, termed soporidine, was shown to bind AtKAI2 and ShHTL7. Micromolar

concentrations of soporidine can block the stimulation of *Striga hermonthica* germination by *rac*-GR24 (Holbrook-Smith et al., 2016).

These strategies may have been improved by greater specificity for parasite KAI2d. Although parasite KAI2d proteins have converged on SL perception with D14, there are substantial differences in their ligand-binding pockets and downstream signaling partners. KAI2d proteins are more similar to AtKAI2 than D14 proteins, of course with obvious differences in their ligand specificities and pockets. Therefore, chemicals that work on D14 or AtKAI2 may not be effective on KAI2d proteins, and vice-versa. The most effective screens for KAI2d agonists and antagonists will likely be based upon direct tests of parasite germination, high-throughput assays for KAI2d activation, or structures of KAI2d proteins. For example, a chemical library screen of 12,000 synthetic molecules for *Striga hermonthica* germination stimulants led to the identification of *N*-arylsulfonylpiperazine as a molecular scaffold that could replace the ABC-ring of SL (Uraguchi et al., 2018). Joining this scaffold to a methylbutenolide D-ring formed SPL7, a specific agonist of ShHTL7 that can trigger *S. hermonthica* germination at femtomolar concentrations. SPL7 has similar potency to the natural SL 5-deoxystrigol, and is at least 100 times more potent than (+)-GR24 on *S. hermonthica* (Uraguchi et al., 2018).

Variability among parasite seed populations, access to parasite seed, and biosafety considerations make parasite germination-based screens particularly challenging. Therefore, there is a need for simple, consistent assays to measure the effects of different compounds on KAI2d activity. Yeast two-hybrid assays between KAI2d proteins and parasite MAX2 or SMAX1 might be an effective primary screen for chemical regulators of KAI2d. Arabidopsis KAI2 is prone to non-specific interactions with MAX2 in yeast two-hybrid, however (Yao et al., 2018a). The middle domains of SMAX1 mediate KAI2 interactions in Arabidopsis, and might provide a clearer interaction assay (Khosla et al., 2020a). Putatively, FRET-based biosensors for SL could also be developed based upon protein-protein interactions with KAI2d, as have already been accomplished for ABA and ABA (Jones et al., 2014; Waadt et al., 2014; Rizza et al., 2017). A functional assay that measures the ability of a parasite KAI2d to target SMAX1 for degradation would be ideal, as the binding or hydrolysis activity of KAI2d proteins for a compound *in vitro* does not always reflect its potency *in vivo*. A highly sensitive and specific assay for SL activity, StrigoQuant, was developed that measures degradation of a ratiometric bioluminescent SMXL6 reporter in Arabidopsis protoplasts (Samodelov et al., 2016). Potentially, a similar system that measures SMAX1 degradation could enable characterization of the ligand preferences and

affinity of a coexpressed parasite KAI2d protein. A ratiometric system that has a similar design principle to StrigoQuant has been used to assay SMAX1 degradation in *Nicotiana benthamiana* leaves (Khosla et al., 2020a; Khosla et al., 2020b). This may be useful as a medium-throughput *in vivo* assay for agonists/antagonists of a KAI2d protein. Another exciting possibility has come from the development of SL biosensors that integrate circular-permutated GFP into DAD2 and ShHTL7 proteins (Chesterfield et al., 2020). A C-terminal fusion of the biosensor to a second fluorescent protein, LSSmOrange, provides an internal control that normalizes for changes in biosensor abundance. These single-protein sensors show a two-fold decrease in GFP fluorescence relative to LSSmOrange in the presence of SLs due to conformational changes that occur in the receptor during SL binding and/or hydrolysis. The cpGFP-ShHTL7 biosensor shows high sensitivity to SLs, with EC_{50} values ranging from 9 nM for *rac*-GR24 to 116 nM for *rac*-5DS (Chesterfield et al., 2020). However, the relative sensitivity of ShHTL7 to *rac*-GR24, *rac*-orobanchol, and *rac*-5DS differs in biosensor assays *versus* YLG competition assays (Tsuchiya et al., 2015; Chesterfield et al., 2020).

Crystal structures of receptors unlock additional approaches to the discovery and design of novel chemical regulators. For example, molecular docking can be used to perform *in silico* screens for potential agonists or antagonists that are refined further through *in vitro* assays and structure-guided optimization. This approach was employed with great success to design a headgroup that dramatically improves the affinity of an ABA receptor agonist, opabactin (Vaidya et al., 2019). In another example, pharmacophore models developed from crystal structures of rice D14 were used to perform *in silico* screening of 4.7 million compounds for potential SL signaling inhibitors (Mashita et al., 2016). Further screening of 61 commercially available compounds for the ability to block GR24-induced yeast two-hybrid interactions between D14 and D53 or SLR1 led to the identification of 2-methoxy-1-naphthaldehyde (2-MN). Interestingly, 2-MN was able to inhibit or partially inhibit germination of *Arabidopsis thaliana* and *Striga hermonthica* in the presence of GR24, suggesting it is a KAI2 antagonist as well (Mashita et al., 2016). If applied against KAI2d structures from parasites, this strategy may prove even more effective for identifying regulators of parasite germination. This is illustrated by molecular docking experiments that examined the placement of 4-bromodebranone (4BD) in the ShHTL5 pocket (Fukui et al., 2017). 4BD was originally identified as a D14 agonist with a simple structure that suppresses tillering, but it only has weak activity on *Striga hermonthica* germination (Fukui et al., 2013). The docking analysis enabled rational design of 4BD analogs that had improved activity and specificity for *Striga* germination (Fukui et al., 2017). A recent

biochemical characterization of 60 ShHTL7 proteins with substitutions in ligand-binding pocket residues enabled the development of a Mutation-dependent Biomacromolecular Quantitative Structure−Activity Relationship (MB-QSAR) model. This model has a strong ability to predict the binding affinities of ShHTL7 mutants and other ShHTL proteins for GR24, and may prove useful for the rational design of new ShHTL regulators (Pang et al., 2020).

Concluding Remarks

The discovery of novel receptors for strigolactones in parasites was an important step toward understanding the dynamic relationship between parasitic Orobanchaceae and their hosts. Further questions about the evolution of this key parasitic adaptation are now raised (see Outstanding Questions). Over the past several years, a remarkable combination of approaches from the disciplines of chemistry, biochemistry, structural biology, evolution, and genetics have rapidly led to detailed mechanistic insights into strigolactone signaling in parasites. With the development of new tools to probe activation of KAI2d proteins *in vitro*, *in vivo*, and *in silico*, there is an unprecedented opportunity to translate these discoveries into better ways to combat parasitic weed infestations and improve crop yields.

Acknowledgements

This work was supported by the National Science Foundation (IOS-1856741) and the USDA National Institute of Food and Agriculture (Hatch project 1023345).

Figure Legends

Figure 1. Chemical structures of representative karrikins, strigolactones, and GR24

KAR_1 and KAR_2 are naturally occurring karrikins in smoke. 5-deoxystrigol (5DS) and 4-deoxyorobanchol are representatives of the strigol-type and orobanchol-type strigolactone classes, respectively, which differ in the stereochemical configuration of the B-C ring junction.

977 *rac*-GR24 is a racemic mixture of a synthetic analog of 5DS and its enantiomer. The D-ring of
978 $GR24^{ent\text{-}5DS}$ has a 2'*S* configuration that has not been found in naturally occurring strigolactones.
979
980 **Figure 2. Representative models of KAI2-SCF^{MAX2} signaling in non-parasitic and parasitic**
981 **plants**
982 In *Arabidopsis thaliana*, KAI2 mediates responses to a putative KAI2 ligand (KL) and an
983 unknown modified form of KAR_1. In many parasitic Orobanchaceae such as *Striga hermonthica*,
984 an increase in gene copies of KAI2 have led to diversified ligand preferences. Cross-species
985 complementation assays suggest that KAI2c proteins preferentially mediate responses to KL,
986 and KAI2i proteins can mediate responses to KARs. However, for unknown reasons obligate
987 parasites do not germinate in response to KAR treatments. A diverse collection of KAI2d
988 proteins function as strigolactone (SL) receptors that can regulate seed germination. Activation
989 of a KAI2 protein induces its association with SCF^{MAX2} and a SMAX1-type SMXL protein (e.g.
990 SMAX1 or SMXL2 in *Arabidopsis thaliana*). The SMXL protein is then polyubiquitinated by
991 SCF^{MAX2} and degraded by the 26S proteasome, triggering downstream responses.
992
993 **Figure 3. Diversification of *KAI2* in parasitic Orobanchaceae**
994 Bar plot of the number of *KAI2* genes and their types detected in Orobanchaceae species and
995 non-parasitic dicots. Obligate parasites require a host to complete their life cycle. Facultative
996 hemiparasites retain photosynthetic capacity and do not require a host for survival.
997 *Lindenberghia philippensis* is a basal non-parasite in the Orobanchaceae. Gene copy estimates
998 are from Conn et al., 2015; Yoshida et al., 2019; and de Saint Germain et al., 2020.
999
1000 **Figure 4. A hypothesis for selective germination responses to strigolactones**
1001 In this model, KAI2d1 and KAI2d2 represent two paralogous receptors that respectively prefer
1002 either strigol-type strigolactone (SL; B-C ring configuration highlighted in purple) or orobanchol-
1003 type SL (B-C ring configuration highlighted in orange) as ligands. For some parasites,
1004 expansion of the KAI2d family may enable responses to a broader range of SLs (left). Activation
1005 of either the KAI2d1 or KAI2d2 receptor by the presence of strigol-type SL or orobanchol-type
1006 SL may cause sufficient SMAX1 degradation to activate germination. This is equivalent to an
1007 OR logic gate. However, the seed of some parasites, such as *Striga gesnerioides*, respond
1008 positively to some SLs but are inhibited by other SLs. This specificity could fine-tune
1009 germination responses to exudates from compatible hosts. In the specialized SL response

hypothesis (right), a dominant-negative mutation (*) causes a KAI2d protein to lose interactions with either MAX2 or SMAX1, but not both, proteins. Here, the mutant KAI2d1* protein can interact with SMAX1 but not MAX2 upon activation. This sequesters SMAX1 and prevents it from being targeted for degradation by other KAI2d-SCF^{MAX2} complexes. Competition between KAI2d1* and fully functional KAI2d proteins that can trigger SMAX1 degradation affects the overall abundance of SMAX1. If SMAX1 levels remain high, germination is blocked. This competition is denoted by versus (VS) and is affected by the presence of SLs that activate KAI2d1* or KAI2d2.

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ADVANCES

- Strigolactone perception by parasite seed is mediated by a clade of neofunctionalized KAI2d proteins that evolved from a receptor that mediates karrikin responses in non-parasitic plants.
- KAI2d proteins use a similar mechanism to perceive strigolactones as D14, which mediates growth responses to strigolactones in non-parasites, but activate different signaling pathways.
- Crystal structure analyses and chemical probes reveal features of KAI2d ligand-binding pockets that contribute to their specificity.

OUTSTANDING QUESTIONS

- How are non-canonical SLs and other lactone molecules that stimulate parasite germination perceived?
- Do KAI2d proteins have adaptive value as SL receptors in facultative hemiparasites that do not show host-activated germination?
- Do some KAI2d proteins negatively regulate germination after detecting specific SLs?
- Is expansion of the KAI2d clade adaptive in the transition to a weedy lifestyle?
- Is host-triggered germination used by obligate parasites of long-lived perennial hosts?
- What are the roles of KAI2c and KAI2i proteins in parasitic plants? Why do KARs not activate germination of S. hermonthica, which expresses KAI2i?
- How does SMAX1 impose seed dormancy, and how has this system become so important to parasite dormancy?
- Which cells in parasite seed control germination? How do parasite seed maintain long-term viability while supporting a system for detecting SLs?

BOX 1. The structure of strigolactones affects their activity in plants

Strigolactones are synthesized from β-carotene via an intermediate molecule, carlactonoic acid (Alder et al., 2012; Seto et al., 2014; Bouwmeester et al., 2020). Carlactonoic acid is converted into either canonical or non-canonical SLs, or both types, depending on the species (Yoneyama et al., 2018b). Canonical SLs are composed of a tricyclic ABC-ring system connected by an enol-ether bridge to a methylbutenolide D-ring. Based upon their stereochemical configuration at the B-C ring juncture, canonical SLs can be subdivided further into orobanchol-type and strigol-type molecules (Figure 1). By contrast, non-canonical SLs have diverse alternatives to the ABC-ring structure, while retaining an enol-ether-linked methylbutenolide D-ring. It is important to note that all known naturally occurring SLs have a 2′*R*-configured D-ring.

BOX 2. How do host-specific germination responses evolve?

Even parasitic weeds with broad host ranges can show selective germination responses to SLs. For example, *Striga hermonthica* seed are much more sensitive to strigol-type SLs than to orobanchol-type SLs (Tsuchiya et al., 2015). By contrast, germination of *Striga gesnerioides* is inhibited by strigol-type SLs and promoted by orobanchol-type SLs (Nomura et al., 2013). This raises the question of how stereoselective germination responses evolved in these species, which have many *KAI2d* paralogs. Gaining a response to a specific SL could be a straightforward process in which a *KAI2d* paralog acquires a mutation(s) that alters its ligand-specificity. Gaining negative germination responses or insensitivity to a SL may be more difficult to achieve, particularly if multiple KAI2d proteins can perceive it. Nonetheless, there is a selective advantage for this to occur when a SL from a non-host plant triggers suicidal germination.

Insensitivity to the disadvantageous SL could emerge through the gradual acquisition of mutations that deactivate the expression or function of all KAI2d that perceive that SL in seed. Alternatively, the same effect could be rapidly achieved through an antimorphic (dominant-negative) mutation of a KAI2d protein that recognizes the disadvantageous SL. Such a mutation would prevent the affected KAI2d protein from interacting with either MAX2 or SMAX1, but not both proteins. This could enable competitive sequestration of SMAX1 or MAX2 from other KAI2d proteins, putatively protecting SMAX1 from degradation and inhibiting germination (Figure 4). Such mutations are plausible, as single amino acid substitutions that affect D14 interactions with MAX2 or D53-type SMXL proteins have been identified (Zhao et al., 2015; Yao et al., 2016; Seto et al., 2019; Lee et al., 2020).

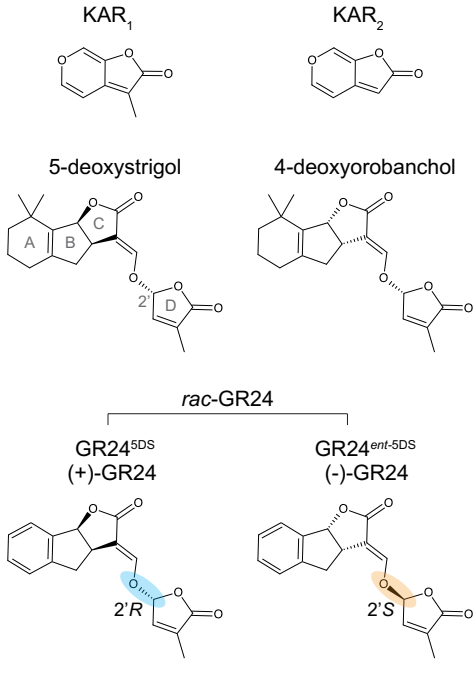

Figure 1. Chemical structures of representative karrikins, strigolactones, and GR24
KAR_1 and KAR_2 are naturally occurring karrikins in smoke. 5-deoxystrigol (5DS) and 4-deoxyorobanchol are representatives of the strigol-type and orobanchol-type strigolactone classes, respectively, which differ in the stereochemical configuration of the B-C ring junction. *rac*-GR24 is a racemic mixture of a synthetic analog of 5DS and its enantiomer. The D-ring of $GR24^{ent\text{-}5DS}$ has a 2'*S* configuration that has not been found in naturally occurring strigolactones.

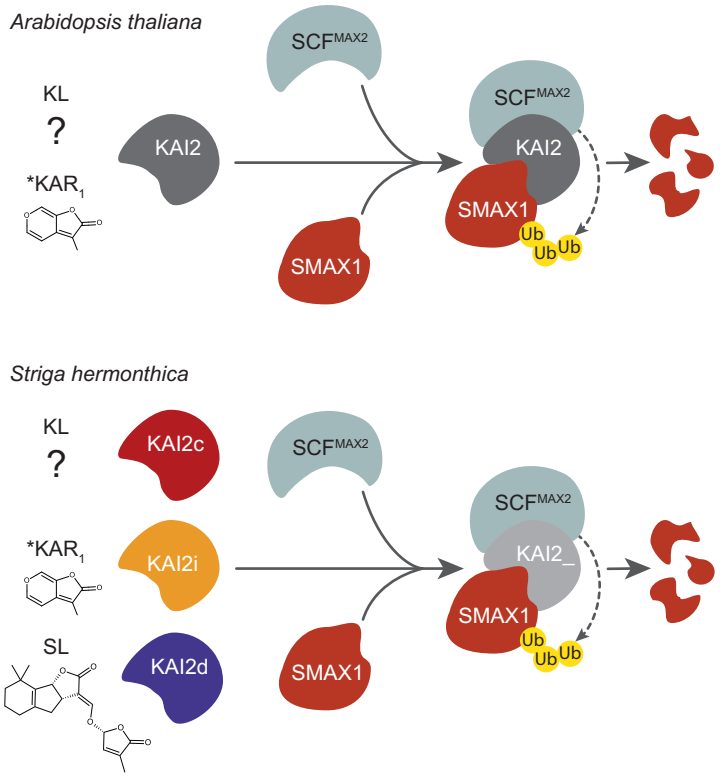

Figure 2. Representative models of KAI2-SCF^{MAX2} signaling in non-parasitic and parasitic plants

In *Arabidopsis thaliana*, KAI2 mediates responses to a putative KAI2 ligand (KL) and an unknown modified form of KAR_1. In many parasitic Orobanchaceae such as *Striga hermonthica*, an increase in gene copies of KAI2 have led to diversified ligand preferences. Cross-species complementation assays suggest that KAI2c proteins preferentially mediate responses to KL, and KAI2i proteins can mediate responses to KARs. However, for unknown reasons obligate parasites do not germinate in response to KAR treatments. A diverse collection of KAI2d proteins function as SL receptors that can regulate seed germination. Activation of a KAI2 protein induces its association with SCF^{MAX2} and a SMAX1-type SMXL protein (e.g. SMAX1 or SMXL2 in *Arabidopsis thaliana*). The SMXL protein is then polyubiquitinated by SCF^{MAX2} and degraded by the 26S proteasome, triggering downstream responses.

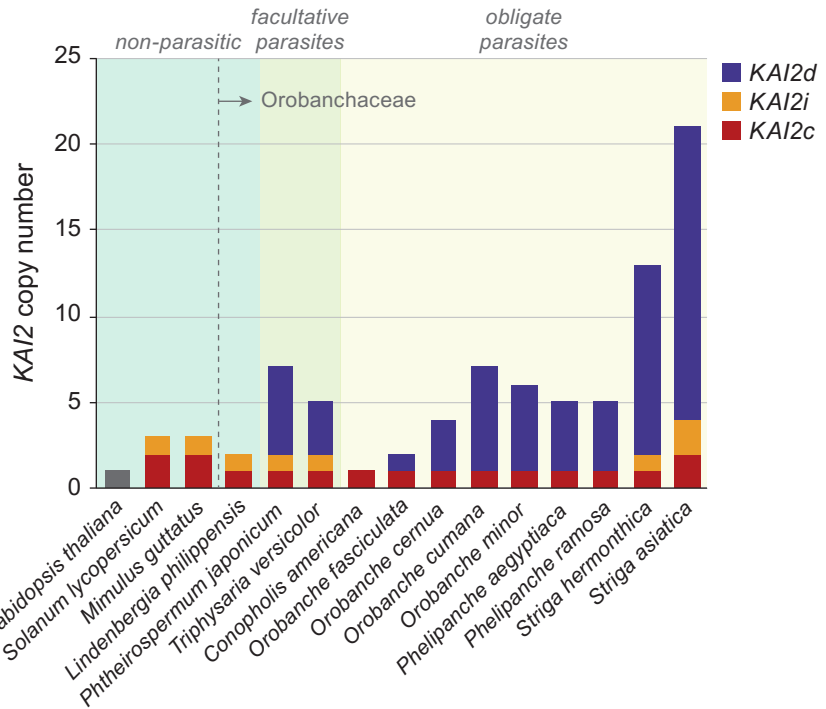

Figure 3. Diversification of *KAI2* in parasitic Orobanchaceae
Bar plot of the number of *KAI2* genes and their types detected in Orobanchaceae species and non-parasitic dicots. Obligate parasites require a host to complete their life cycle. Facultative hemiparasites retain photosynthetic capacity and do not require a host for survival. *Lindenberghia philippensis* is a basal non-parasite in the Orobanchaceae. Gene copy estimates are from Conn et al., 2015; Yoshida et al., 2019; and de Saint Germain et al., 2020.

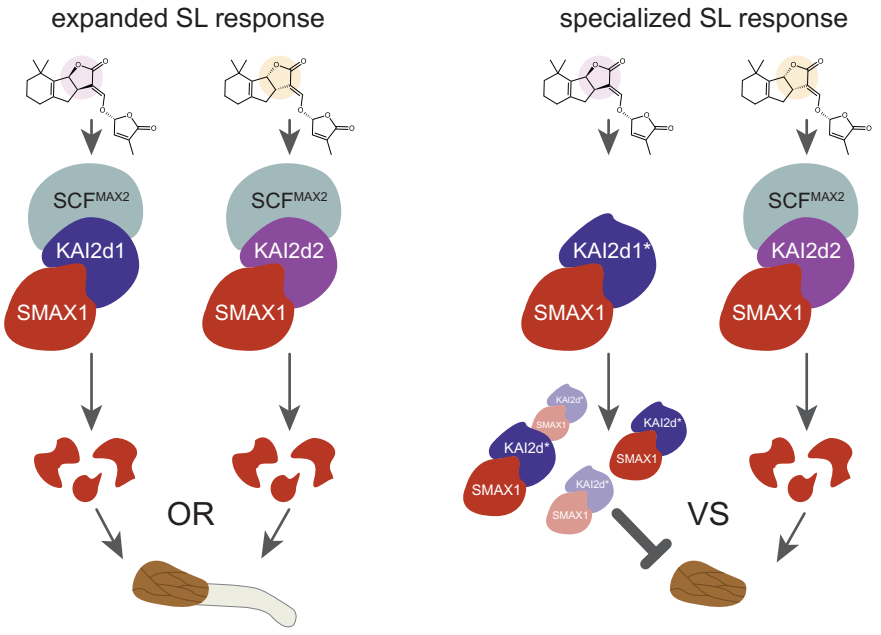

Figure 4. A hypothesis for selective germination responses to strigolactones In this model, KAI2d1 and KAI2d2 represent two paralogous receptors that respectively prefer either strigol-type SL (B-C ring configuration highlighted in purple) or orobanchol-type SL (B-C ring configuration highlighted in orange) as ligands. For some parasites, expansion of the KAI2d family may enable responses to a broader range of SLs (left). Activation of either the KAI2d1 or KAI2d2 receptor by the presence of strigol-type SL or orobanchol-type SL may cause sufficient SMAX1 degradation to activate germination. This is equivalent to an OR logic gate. However, the seed of some parasites, such as *Striga gesnerioides*, respond positively to some SLs but are inhibited by other SLs. This specificity could fine-tune germination responses to exudates from compatible hosts. In the specialized SL response hypothesis (right), a dominant-negative mutation causes a KAI2d protein to lose interactions with either MAX2 or SMAX1, but not both, proteins. Here, KAI2d1* can interact with SMAX1 but not MAX2 upon activation. This sequesters SMAX1 and prevents it from being targeted for degradation by other KAI2d-SCFMAX2 complexes. Competition between KAI2d1* and fully functional KAI2d proteins that can trigger SMAX1 degradation affects the overall abundance of SMAX1. If SMAX1 levels remain high, germination is blocked. This competition is denoted by versus (VS) and is affected by the presence of SLs that activate KAI2d1* or KAI2d2.

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