Karrikin perception and signalling

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SUMMARY

Karrikins are a class of butenolide compounds found in smoke that were first identified as seed germination stimulants for fire-following species. Early studies of karrikins classified the germination and post-germination responses of many plant species, and investigated crosstalk with plant hormones that regulate germination. The discovery that *Arabidopsis thaliana* responds to karrikins laid the foundation for identifying mutants with altered karrikin responses. Genetic analysis of karrikin signalling revealed an unexpected link to strigolactones, a class of carotenoid-derived plant hormones. Substantial progress has since been made toward understanding how karrikins are perceived and regulate plant growth, in no small part due to advances in understanding strigolactone perception. Karrikin and strigolactone signalling systems are evolutionarily related and retain a high degree of similarity. There is strong evidence that karrikins (KARs) are natural analogues of an endogenous signal(s), KAI2 ligand (KL), which remains unknown. KAR/KL signalling regulates many developmental processes in plants including germination, seedling photomorphogenesis, and root and root hair growth. KAR/KL signalling also affects abiotic stress responses and arbuscular mycorrhizal symbiosis. Here we summarise the current knowledge of KAR/KL signalling, and discuss current controversies and unanswered questions in this field.

INTRODUCTION

Fires have profound effects on local ecosystems, causing the immediate destruction of plants and often long-term changes in plant community composition. One way that plants regrow after fire is through breaking the dormancy of seeds buried in soil. The most dramatic examples of this are found among fire ephemeral, or pyroendemic, plants that only emerge after fire, sometimes after many years of absence. In the early 1990s, it was discovered that chemical cues from smoke, rather than heat itself, were sufficient to activate seed germination of many fire-following species (De Lange & Boucher, 1990; Keeley & Pausas, 2018). Application of aerosol smoke or smoke-water solutions to bushland soil can cause dramatic increases in the number of seedlings and different species that later emerge. At least 1200 species have positive germination responses to smoke or smoke-water (Sweedman & Merritt, 2006; Dixon *et al.*, 2009; Jefferson *et al.*, 2014).

Several germination-promoting compounds have been found in the highly complex mixture of chemicals that is smoke, including NO_2, glyceronitrile, and karrikins (Keeley & Fotheringham, 1997; Flematti *et al.*, 2004, 2011; van Staden *et al.*, 2004; Keeley & Pausas, 2018). Karrikins, so named for “karrik”, an Aboriginal Noongar word for smoke, are a family of small, water-soluble, butenolide compounds that are potent germination stimulants for many fire-following species (Dixon *et al.*, 2009; Flematti *et al.*, 2009; Nelson *et al.*, 2012) (Box 1). In some cases, low nanomolar concentrations of karrikins are effective at triggering germination (Flematti *et al.*, 2004, 2007).

Notably, karrikin responses are not restricted to germination, nor to species that are endemic to fire-prone ecosystems. KAR_1 improves the germination, seedling vigour, and stress tolerance of many crops (Antala *et al.*, 2019). Genetic studies in Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), *Lotus japonicus*, and *Brachypodium distachyon* have shown that the karrikin pathway also controls photomorphogenic seedling growth, mesocotyl elongation in the dark, anthocyanin abundance, cuticular wax deposition, abiotic and drought stress tolerance, leaf shape, root hair density and elongation, root skewing, and symbiosis with arbuscular mycorrhizal (AM) fungi (Figure 1) (Nelson *et al.*, 2010; Waters *et al.*, 2012; Stanga *et al.*, 2013, 2016; Soundappan *et al.*, 2015; Gutjahr *et al.*, 2015; Li *et al.*, 2017, 2020; Wang *et al.*, 2018; Swarbreck *et al.*, 2019; Choi *et al.*, 2020; Zheng *et al.*, 2020; Carbonnel *et al.*, 2020a; Bursch *et al.*, 2021; Villaécija-Aguilar *et al.*, 2022; Meng *et al.*, 2022; Feng *et al.*, 2022).

Genetics to the rescue: identifying the elements of karrikin signalling

The discovery that karrikins promote germination and seedling photomorphogenesis of *Arabidopsis thaliana* opened the door for understanding how karrikins are perceived and regulate plant growth (Nelson *et al.*, 2009, 2010). A forward genetic screen revealed that karrikin responses in Arabidopsis require the F-box protein MORE AXILLARY GROWTH2 (MAX2, known as DWARF3 (D3) in rice) (Nelson *et al.*, 2011). Several plant hormone signalling systems, including those of auxin, jasmonate, and gibberellin, involve ligand-activated proteolysis that is mediated by F-box proteins acting in SCF-type (Skp1, Cullin, F-box) E3 ubiquitin ligase complexes (Blázquez *et al.*, 2020). Thus, the discovery that an F-box protein was involved in karrikin responses was not altogether surprising. What was unexpected, however, was that MAX2/D3 is also required for strigolactone signalling, but karrikins and strigolactones mostly affect plant growth and development in different ways (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Nelson *et al.*, 2011). This implied that there

must be a way for SCF^{MAX2}-mediated signalling to discriminate between karrikins and strigolactones during signal perception (input) and activation of different downstream responses (output).

Progress on the input front was greatly assisted by the earlier characterisation and mapping of the strigolactone-insensitive *dwarf14* (*d14*) mutant of rice (Arite *et al.*, 2009). At the time, the function of the α/β-hydrolase encoded by *D14* was not understood, beyond its requirement for strigolactone response. However, it was clear that angiosperms had two clades of genes with similarity to *D14*: one was defined by *D14* itself, while the second clade of unknown function was represented by another rice gene named *D14-LIKE* (Arite *et al.*, 2009). In 2012, two mutant alleles of the Arabidopsis orthologue of *D14-LIKE*, also known as *HYPOSENSITIVE TO LIGHT* (*HTL*), were discovered to be karrikin-insensitive, but strigolactone-responsive; thus the gene was named *KARRIKIN INSENSITIVE2* (*KAI2*) (Sun & Ni, 2011; Waters *et al.*, 2012). (Note that *HTL* nomenclature is typically used for *KAI2* homologues in the parasitic weed *Striga hermonthica* (Tsuchiya *et al.*, 2015; Toh *et al.*, 2015), whereas *D14-LIKE* (*D14L*) nomenclature is only used in rice.) By contrast, an Arabidopsis *d14* mutant is strigolactone-insensitive and karrikin-responsive, whereas *max2* shows the combined phenotypes of *kai2* and *d14* (Waters *et al.*, 2012; Soundappan *et al.*, 2015). Thus, *D14* and *KAI2* contribute to discrimination of strigolactones and karrikins. An orthologue of *D14* in petunia, *DECREASED APICAL DOMINANCE2* (*DAD2*), provided the first evidence that D14 proteins are SL receptors (Hamiaux *et al.*, 2012). This role has since been validated in many species (Yao *et al.*, 2016; de Saint Germain *et al.*, 2016; Waters *et al.*, 2017).

The most comprehensive phylogenetic analysis of *KAI2* and *D14* homologues to date has resulted in a revised nomenclature to accommodate the complexity and likely evolutionary relationships in this family (Box 2) (Bythell-Douglas *et al.*, 2017). Note that the DLK23 clade, represented by *D14-LIKE2* (*DLK2*) in Arabidopsis, is actually more similar than *KAI2* to *D14* than is *KAI2*. True *D14* orthologues are restricted to seed plants, but *KAI2* orthologues are present in all land plants and some algal ancestors. This distribution implies that *D14* evolved from a *KAI2*-like ancestral sequence.

On the output side of MAX2, a screen for mutations that suppress *max2* phenotypes in seed and seedlings uncovered a recessive allele of *SUPPRESSOR OF MAX2 1* (*SMAX1*) (Stanga *et al.*, 2013). *SMAX1* is one of eight *SMAX1-LIKE* (*SMXL*) genes in Arabidopsis, a family that originated as four major types in the angiosperm lineage: *SMAX1* (*SMAX1* and *SMXL2* in Arabidopsis), *SMXL39* (*SMXL3* in Arabidopsis), SMXL4 (*SMXL4* and *SMXL5* in Arabidopsis), and *SMXL78* (*SMXL6*, *SMXL7*, and *SMXL8* in Arabidopsis). Fortunately for the success of a suppressor screen, *SMAX1* is the primary regulator of germination and seedling growth downstream of *MAX2* in Arabidopsis, so the *smax1* single mutant shows obvious phenotypes. *SMXL2* is partially redundant with *SMAX1*, primarily at the seedling stage (Stanga *et al.*, 2016). In combination, recessive, loss-of-function alleles of *smax1* and *smxl2* are epistatic to *max2* and impart reduced seed dormancy, shortened seedling hypocotyls, and transcriptional patterns opposite to *kai2* and *max2*; in other words, a phenotype consistent with a constitutive karrikin response.

Genetic screens for suppressors of the excess shoot branching phenotype of *max2* had been attempted previously, but in retrospect this approach was unlikely to work because of redundancy among the *SMXL* family members that regulate shoot architecture (Soundappan *et al.*, 2015; Wang *et al.*, 2015). This issue was bypassed by identification of a dominant, gain-

of-function mutant in rice, *dwarf53* (*d53*), that mimicked SL-insensitive mutants. D53, an orthologue of SMXL6, SMXL7, and SMXL8 in Arabidopsis, was the first SMXL protein demonstrated to be a target of D14-SCF^{MAX2} in response to strigolactone (Jiang *et al.*, 2013; Zhou *et al.*, 2013). This role was later validated for SMXL6, SMXL7, and SMXL8 through biochemical assays and suppression of strigolactone-associated *max2* phenotypes by loss-of-function *smxl* alleles (Soundappan *et al.*, 2015; Wang *et al.*, 2015; Liang *et al.*, 2016). The transcription factor BRASSINOSTEROID INSENSITIVE1-ETHYL METHANESULFONATE-SUPPRESSOR1 (BES1) was also proposed to be a target of MAX2 involved in strigolactone regulation of shoot branching, but this idea has been challenged by further genetic analysis (Wang *et al.*, 2013; Bennett *et al.*, 2016).

SMAX1 and SMXL2 are targeted for ubiquitination and degradation by SCF^{MAX2} through activation of KAI2 and regulate karrikin-associated traits (Wang *et al.*, 2020b; Khosla *et al.*, 2020a; Zheng *et al.*, 2020; Park *et al.*, 2022). For these reasons, SMAX1 and SMXL2 are sometimes described as negative regulators of karrikin signalling that operate downstream of MAX2. However, it is more accurate to consider SMXL proteins similarly to DELLA proteins. Although DELLA proteins are targeted by gibberellin signalling for proteolysis, it is better to think of them as signalling hubs rather than repressors of gibberellin responses. DELLAs control many growth and defence transcriptional programs and are in turn post-translationally regulated by multiple gibberellin-independent mechanisms (Blanco-Touriñán *et al.*, 2020). Similarly, SMAX1 and SMXL2 protein abundance is regulated by karrikin signalling, but not exclusively (Khosla *et al.*, 2020a; Park *et al.*, 2022; Kim *et al.*, 2022). Among other examples, in some circumstances SMAX1 and SMXL2 can be targeted for degradation by D14-mediated strigolactone signalling (Wang *et al.*, 2020b; Li *et al.*, 2022).

Signal perception and hydrolysis by D14 and KAI2

Our knowledge of karrikin signalling is closely interwoven with that of strigolactones because the mechanisms are broadly the same, and progress on one front has helped inform the other. As such, neither can really be discussed in isolation from the other. D14 and KAI2 are α/β-hydrolases with a dual receptor-enzyme function. A core of α-helices and β-sheets (the "α/β fold") is linked to a cap composed of two V-shaped pairs of α-helices. Between these two domains sits a conserved catalytic triad of Ser, His and Asp residues at the base of a hydrophobic substrate-binding pocket (Figure 2). Both D14 and KAI2 are capable of hydrolysing generic substrates such as *para*-nitrophenyl acetate. Both proteins also hydrolyse the strigolactone analogue GR24, albeit with preferences for different stereoisomers that compose the typical racemic GR24 (*rac*-GR24) mixture (Box 1) (Sun & Ni, 2011; Hamiaux *et al.*, 2012; Waters *et al.*, 2015b; de Saint Germain *et al.*, 2016). Incubation of purified D14 and KAI2 with bioactive compounds, such as GR24, leads to a decrease in the melting temperature of the proteins, as detected by fluorescence-based assays such as differential scanning fluorimetry (DSF). These changes in thermostability suggest that the signalling mechanism involves a conformational change in the receptor into an activated state, which is supported by structural data (see below). Receptor variants with mutated catalytic residues do not respond with a decrease in thermostability, which implies either that ligand hydrolysis is a prerequisite for signalling, or that ligand interaction with one or more catalytic residues is necessary to initiate the conformational change (Hamiaux *et al.*, 2012; Waters *et al.*, 2015b; Seto *et al.*, 2019). Notably, the hydrolysis reaction results in covalent modification of the catalytic triad, which has been detected by crystallography and mass spectrometry. The

cleaved butenolide ring can be opened to bridge the Ser and His residues, forming a covalently linked intermediate molecule (CLIM), or can be bound to the Ser or His residues alone (Yao *et al.*, 2016; de Saint Germain *et al.*, 2016; Guercio *et al.*, 2022). Quantum mechanics/molecular mechanics simulations have revealed that all of these reported modifications are likely to occur, but CLIM is predicted to be the most energetically favourable and dominant form (Chen & Shukla, 2022).

The precise significance of ligand hydrolysis is contested. On the one hand, mutation of the catalytic serine or histidine renders D14 and KAI2 non-functional as enzymes and receptors. Such mutant proteins have abolished hydrolytic activity, do not interact with SCF^{MAX2} or their target SMXL proteins, and are not themselves degraded after ligand perception (Hamiaux *et al.*, 2012; Jiang *et al.*, 2013; Waters *et al.*, 2015a,b; Hu *et al.*, 2017; Seto *et al.*, 2019; Lee *et al.*, 2020; Khosla *et al.*, 2020a). Furthermore, non-hydrolysable substrates are much less biologically active, or even antagonistic (Takeuchi *et al.*, 2018; Uraguchi *et al.*, 2018). However, mutating the catalytic Asp residue in Arabidopsis D14 prevents GR24 hydrolysis without abolishing strigolactone signalling in transgenic plants (Seto *et al.*, 2019). Instead, Arabidopsis $d14^{D218A}$ transgenic lines were reported to have slightly increased sensitivity to strigolactones (Seto *et al.*, 2019). These findings imply that hydrolysis is simply a mechanism for inactivation of the ligand after signalling, and a resetting of the receptor.

However, an unrelated carboxylesterase enzyme has since been identified in Arabidopsis that hydrolyses strigolactones and related analogues with much greater catalytic efficiency than D14 (Xu *et al.*, 2021), which implies that there exists an independent route for SL breakdown. Furthermore, both D14 and KAI2 are degraded following signalling (Chevalier *et al.*, 2014; Waters *et al.*, 2015a; Hu *et al.*, 2017; Sanchez *et al.*, 2018; Khosla *et al.*, 2020b; Li *et al.*, 2022), albeit over a timescale of minutes to hours, which raises doubts about the benefit of resetting the receptor by hydrolysing the ligand. If the aspartic acid is only necessary for ligand inactivation, then why are there no reported D14 or KAI2 homologues with a substituted aspartic acid residue? Perhaps an intact catalytic triad helps to maintain the overall protein conformation and stability; or, perhaps $d14^{D218A}$ is predisposed to adopt the active conformational state, and ligand binding is sufficient to complete the transition. This possibility is consistent with the observation that the D218 residue is located on a flexible loop that is displaced away from the ligand-binding pocket during strigolactone perception (Yao *et al.*, 2016; Chen *et al.*, 2021, 2022). Alternatively, immediate ligand hydrolysis by the receptor might provide short-term, highly localised control over signalling that optimises sensitivity over a narrow range of substrate concentrations. Meanwhile, receptor degradation and strigolactone catabolism might serve as longer term homeostatic mechanisms, especially in response to external environmental cues, and perhaps in a tissue or organ-specific manner (Xu *et al.*, 2021).

The canonical mechanism for strigolactone perception proposes nucleophilic attack upon the butenolide carbonyl group by the catalytic serine residue of D14, which opens up the butenolide ring (Yao *et al.*, 2016). This event is followed by the covalent attachment of a 96-Da butenolide derivative to the catalytic histidine, as detected by mass spectrometry (Yao *et al.*, 2016; de Saint Germain *et al.*, 2016). This mechanism is supported by the observation of a covalently-linked intermediate molecule (CLIM) trapped in the pocket of D14 during co-crystallisation with GR24 and D3 (Yao *et al.*, 2016), although the exact identity and location of CLIM has faced some scrutiny (Carlsson *et al.*, 2018; Bürger & Chory, 2020). Further evidence

comes from the crystallographic observation of a bond between the catalytic serine and the open butenolide ring of a non-hydrolysable strigolactone analogue (Takeuchi *et al.*, 2018).

Until very recently, it was assumed that substrate recognition and hydrolysis by KAI2 was essentially the same as for D14, but direct experimental evidence was lacking. We now know that Arabidopsis KAI2 and several homologues from *Physcomitrium patens* form covalent adducts with a 96-Da mass when incubated with the 2′*S*-configured compounds GR24$^{ent\text{-}5DS}$ and *ent*-5-deoxystrigol (Bürger *et al.*, 2019; Lopez-Obando *et al.*, 2021); in the latter case, these adducts were mapped to the active site histidines, as seen with D14. Even more compelling evidence has come from X-ray crystallography studies of PsKAI2B from pea, which revealed the probable presence of 5-hydroxy-3-methylbutenolide attached to the catalytic serine (Guercio *et al.*, 2022). This compound probably represents the first reaction intermediate following nucleophilic attack on the butenolide carbonyl and is probably highly transient, because the presumably more stable 96-Da covalent adduct was again localised to the catalytic histidine by mass spectrometry (Guercio *et al.*, 2022). This model is further supported by molecular dynamics and quantum mechanical free energy simulations that favour the butenolide carbonyl as the initial target (Chen & Shukla, 2022). Not only do these findings support the likely catalytic mechanism of KAI2 upon butenolide ligands, but they also refine the previously proposed reaction mechanism for D14 (Yao *et al.*, 2016; de Saint Germain *et al.*, 2016). Together, these studies make it highly likely that both types of butenolide receptors operate with the same mode of action.

KAI2 is probably not a karrikin receptor

Genetic studies have clearly shown that KAI2 is necessary for karrikin responses (Waters *et al.*, 2012). In addition, KAI2 has been shown to bind KAR_1 with a broad range of affinities through several *in vitro* techniques including isothermal calorimetry, equilibrium microdialysis, heteronuclear single quantum coherence, and dye–based thermal denaturation (Kagiyama *et al.*, 2013; Guo *et al.*, 2013; Xu *et al.*, 2016, 2018; Lee *et al.*, 2018; Bürger *et al.*, 2019). This has led to the understandable conclusion in the literature that KAI2 is a karrikin receptor, but we no longer consider this to be entirely accurate.

We argue that the ability of KAI2 to bind a molecule does not necessarily mean that the molecule can activate KAI2 and initiate downstream signal transduction. Several observations (Box 3) have led us and others to conclude that karrikins must be metabolised *in vivo* before perception by KAI2 can occur (Waters *et al.*, 2015b; Xu *et al.*, 2018; Wang *et al.*, 2020b; Khosla *et al.*, 2020a). The mechanism of SL perception by D14 and GR24$^{ent\text{-}5DS}$ perception by PsKAI2B suggests that a hydrolyzable butenolide moiety, which covalently modifies one or more catalytic triad residues, may be a common feature of KAI2 agonists (Yao *et al.*, 2016; de Saint Germain *et al.*, 2016; Guercio *et al.*, 2022). If so, perhaps the conversion of karrikin into a bioactive molecule involves the formation of a cleavable butenolide ring.

Furthermore, we propose that the typical function of KAI2 is not to mediate karrikin responses. For many plants, such as Arabidopsis (*Arabidopsis thaliana*) and lettuce (*Lactuca sativa*), the adaptive value of a karrikin response mechanism is unclear. Perhaps fire played a more important role in the ecology of some plant lineages in past eras and a karrikin signalling system has been maintained by chance in extant descendants. Alternatively, we favour the idea that karrikin metabolites fortuitously activate KAI2, which normally recognizes an

endogenous plant growth regulator(s) known as KAI2 ligand (KL). This is conceptually similar to a drug or small molecule in a chemical library (in this case, smoke) that can modulate the activity of a protein. Karrikin-activated germination could have evolved in some fire followers through *KAI2* duplication and selection for enhanced karrikin sensitivity over KL in one paralogue (Martinez *et al.*, 2022).

Although KL has not yet been identified (Box 4), several observations have led to a widespread expectation that it exists (Flematti *et al.*, 2013; Conn & Nelson, 2015; Machin *et al.*, 2020; Guercio *et al.*, 2022; Bonhomme & Guillory, 2022). First, *kai2* and *max2* mutants are not only karrikin-insensitive, but show seed and seedling phenotypes that are opposite to karrikin-induced responses (Nelson *et al.*, 2011; Waters *et al.*, 2012; Villaécija-Aguilar *et al.*, 2019). For example, karrikins promote germination, whereas *kai2* and *max2* have enhanced seed dormancy. This implies that either KAI2-SCF^{MAX2} has ligand-independent, as well as karrikin-enhanced, signalling activity, or that responses to an endogenous signal have been lost in *kai2* and *max2* (Nelson *et al.*, 2011; Waters *et al.*, 2012). Arguing against constitutive, ligand-independent KAI2 activity, 25-fold overexpression of *KAI2* enhanced the sensitivity of Arabidopsis seedlings to karrikin application without affecting growth in the absence of treatment (Waters & Smith, 2013). Second, the strict conservation of a catalytic triad in KAI2 proteins implies that substrate hydrolysis is important for their function, as it is for the homologous strigolactone receptor D14 (Bythell-Douglas *et al.*, 2017). Indeed, the catalytic Ser is required for the enzymatic and signalling functions of KAI2 (Waters *et al.*, 2014, 2015a,b). If KAI2 signalling were primarily ligand-independent (e.g. KAI2 has constitutive protein-protein interactions with SCF^{MAX2} and its targets, SMAX1/SMXL2), then purifying selection to maintain the triad residues seems less likely. Third, aqueous phase extracts from Arabidopsis leaves stimulate expression of a transcriptional reporter of KAR signalling in a *KAI2*-dependent manner, suggesting the presence of KL (Sun *et al.*, 2016). Finally, KAI2 proteins in many species, such as Arabidopsis, *Brassica tournefortii*, rice, pea, and *Lotus japonicus*, can rescue an Arabidopsis *kai2* mutant and restore responses to karrikins, implying these receptors have the flexibility to respond to both KL and karrikins (Waters *et al.*, 2012; Sun *et al.*, 2020; Carbonnel *et al.*, 2020b; Guercio *et al.*, 2022). However, among the *KAI2* paralogs that have arisen from gene duplication events in asterids and the parasitic Orobanchaceae are several examples of proteins that appear to have subfunctionalized responses to KL and karrikins. Some *KAI2* variants can rescue Arabidopsis *kai2* but do not confer responses to karrikins, while others cause little or no rescue of *kai2* but show sensitive responses to karrikins (Conn & Nelson, 2015; Conn *et al.*, 2015; Martinez *et al.*, 2022). Therefore, perception of KL and karrikins by KAI2 can be genetically separated.

The KAR/KL signalling pathway is found in lycophytes and bryophytes as well as angiosperms (Mizuno *et al.*, 2021; Lopez-Obando *et al.*, 2021; Kodama *et al.*, 2022; Bonhomme & Guillory, 2022). In one case, a *KAI2* gene from the lycophyte *Selaginella moellendorffii* has been shown to partially rescue several Arabidopsis *kai2* phenotypes, although it does not confer responses to karrikins or other known KAI2 agonists. It requires an active catalytic triad to do so (Waters *et al.*, 2015b). This suggests KL signalling was present in an early ancestor of land plants. Genetic studies in the bryophyte *Marchantia polymorpha* have shown that the KAI2-SCF^{MAX2}-SMXL pathway regulates thallus growth, thallus orientation, and gemma cell proliferation (Mizuno *et al.*, 2021). KAI2a in *M. polymorpha* is putatively responsive to GR24$^{ent\text{-}5DS}$, similar to many KAI2 proteins in angiosperms. In contrast to rice, however, *KAI2* and *MAX2* are not required for AM symbiosis in *Marchantia paleacea*, suggesting this role emerged in vascular

plant lineages (Kodama *et al.*, 2022). In terms of the origins of SL perception, *M. paleacea* produces a non-canonical SL, bryosymbiol, that stimulates symbiosis with AM fungi. Interestingly, bryosymbiol-deficient mutants lack obvious phenotypes other than reduced mycorrhization (Kodama *et al.*, 2022). In addition, *KAI2* and *MAX2* (there is no *D14*) are not involved in SL perception in *M. paleacea*. Therefore, the earliest roles for SL may have been symbiotic rather than hormonal; putatively, SL was later co-opted to act as a hormone in the vascular plant lineage through adaptation of the KAR/KL pathway.

Ligand preferences of KAI2

Collectively, the KAI2 protein family mediates perception of multiple chemical stimuli. The molecular basis of the different ligand preferences that have evolved among KAI2 proteins has been a subject of intense interest. Initially, the significance of stereochemistry in substrate selectivity by KAI2 and D14 was not fully appreciated, leading to misattribution of some *rac*-GR24 effects as strigolactone responses. It is now known that in Arabidopsis, KAI2 has a strong preference for GR24$^{ent\text{-}5DS}$, whereas D14 has a strong preference for GR24^{5DS} (Scaffidi *et al.*, 2014). These preferences are not absolute under all contexts (Villaécija-Aguilar *et al.*, 2019), but have proven to be generally robust across a broad taxonomic range of KAI2 and D14 proteins and multiple assays, both biochemical and physiological (Sun *et al.*, 2020; Khosla *et al.*, 2020a; Carbonnel *et al.*, 2020b; Lopez-Obando *et al.*, 2021; Guercio *et al.*, 2022). There is however a notable exception to this pattern: many evolutionarily divergent KAI2 proteins from parasitic weeds in the Orobanchaceae have evolved to perceive host-derived strigolactones, and therefore show the greatest sensitivity to GR24^{5DS} and natural strigolactones (Conn & Nelson, 2015; Conn *et al.*, 2015; Tsuchiya *et al.*, 2015; Toh *et al.*, 2015; Nelson, 2021; Wang *et al.*, 2021a; Arellano-Saab *et al.*, 2021).

Although many smoke-responsive species respond robustly to KAR_1 (Flematti *et al.*, 2007; Sun *et al.*, 2020; Martinez *et al.*, 2022), many other species (for example, *Arabidopsis thaliana* and *Brachypodium distachyon*) show a preference for KAR_2 (Nelson *et al.*, 2009, 2010; Waters *et al.*, 2012; Meng *et al.*, 2022). Karrikin preference has been investigated for KAI2 homologues from several species (Table 1). Interestingly, *Lotus japonicus* seedlings show tissue-specific differences in responses to KAR_1, KAR_2, and *rac*-GR24 (Carbonnel *et al.*, 2020b). This raises the possibility that KAR/KL perception can be affected by cellular context, for example through modulation of signal transport, metabolism, expression of *KAI2* variants, and SMAX1 stability.

Several studies have investigated the molecular basis for karrikin preferences with a focus on *KAI2* (Xu *et al.*, 2016; Sun *et al.*, 2020; Carbonnel *et al.*, 2020b; Guercio *et al.*, 2022; Martinez *et al.*, 2022). One commonly used approach has been to identify the various KAI2 proteins and assess their karrikin preference in cross-species complementation assays by expressing them in an Arabidopsis *kai2* or *kai2 d14* null mutant background. Because karrikins probably require metabolism to become bioactive ligands for KAI2, *in vitro* assays for ligand-receptor interactions can be misleading (Box 3). Examining KAI2 activity *in vivo* through transgenics overcomes this problem. Another common approach has been the use of structural homology modelling to identify residues near the KAI2 ligand-binding pocket that might influence ligand selectivity. This is most effective when coupled with functional tests of candidate residues.

One study investigated karrikin perception in *Brassica tournefortii*, an invasive weed of Mediterranean-type biomes that thrives in fire-prone environments thanks in part to stimulation

of germination by KAR_1. Whole genome triplication has resulted in three *KAI2* paralogues. Two, *BtKAI2a* and *BtKAI2b*, encode functional KAI2 proteins but confer alternate karrikin preferences to transgenic Arabidopsis (Sun *et al.*, 2020). Residue-swapping experiments identified two leucine residues (Leu98 and Leu191) in BtKAI2b that are sufficient to specify preference for KAR_1 over KAR_2 in Arabidopsis; of the two, Leu98 is primarily responsible for the effect. Although rare among a sample of nearly 500 angiosperm KAI2 sequences, the two leucines are more frequently observed together than expected by chance, suggesting the possibility of functional co-dependency (Sun *et al.*, 2020).

Selective karrikin responses are also found in legumes. A gene duplication event prior to the diversification of legumes produced two *KAI2* paralogues in *Lotus japonicus*, soybean (*Glycine max*), barrel medic (*Medicago truncatula*), and pea (*Pisum sativum*) (Carbonnel 2020; Guercio 2022). *LjKAI2b* from *L. japonicus* confers a preference for KAR_1 over KAR_2 in transgenic Arabidopsis, whereas *LjKAI2a* is ambivalent; interestingly, *LjKAI2b* is unable to mediate responses to either GR24 enantiomer, although *LjKAI2a* could (Carbonnel *et al.*, 2020b). Structure-guided inspection of the differences between both proteins and their corresponding orthologues in other legumes identified three distinguishing residues. Although the effect of these residues on karrikin specificity *in planta* was not determined, a tryptophan-to-phenylalanine substitution at position 158 accounts for most of the differential response of each protein to $GR24^{ent\text{-}5DS}$, both *in vitro* and in Arabidopsis (Carbonnel *et al.*, 2020b). Surprisingly, the W158 residue in LjKAI2b, which renders it unresponsive to $GR24^{ent\text{-}5DS}$, is relatively rare among angiosperms and is not found in other legume KAI2 proteins in the same clade. Notably, the orthologous PsKAI2B from *P. sativum*, which was successfully crystallised with a $GR24^{ent\text{-}5DS}$ reaction intermediate (Guercio *et al.*, 2022), has a canonical phenylalanine at the same position. In *P. sativum*, *DLK2* transcript abundance increases only in response to KAR_1 and not to KAR_2. Although it is not quite clear whether only one or both *KAI2* paralogues contribute to this preference, *PsKAI2b* appears to account for most of the KAR_1 response (Guercio *et al.*, 2022). As such, it is likely that the same *KAI2* orthologue confers a preference for KAR_1 over KAR_2 in legumes, but the specific residues responsible for this difference remain unresolved.

Most recently, the basis for a strong and sensitive response to KAR_1 in lettuce (*Lactuca sativa* cv. “Grand Rapids”) – which guided the first isolation of KAR_1 from smoke-water (Flematti *et al.*, 2004) – was elucidated. The lettuce genome contains two *KAI2* paralogs. *LsKAI2b* transcripts are several-fold more abundant in dry achenes than *LsKAI2a*. *LsKAI2b* also confers highly specific and sensitive KAR_1 responses as a transgene in Arabidopsis, but *LsKAI2a* does not (Martinez *et al.*, 2022). Several residues that may contribute to ligand-specificity were identified through comparisons of the predicted ligand-binding pockets of KAI2 proteins with known or suspected preferences for KAR_1 to in-species KAI2 paralogues that do not. A broader comparison of KAI2 proteins in asterids revealed that five of these pocket sites (positions 96, 124, 139, 161, and 190) appear to be co-evolving among two major groups. KAI2 proteins with a Tyr124 residue are broadly present among asterids, whereas KAI2 proteins with a Phe124 substitution, as in LsKAI2b, are less common. The emergence of LsKAI2b in lettuce is likely to have occurred independently within the Asterales lineage, as a similar Phe124-type of KAI2 protein was not observed in 36 related species. An analysis of Arabidopsis KAI2 variants with one or more substitutions at positions 96, 124, 139, and 161 showed that position 124 is an important determinant of KAR_2 responsiveness, while

substitutions at the other positions can affect KAR_1 or *rac*-GR24 response in complex ways (Martinez *et al.*, 2022).

Addressing KAI2 ligand specificity from a different angle, Arellano-Saab *et al*. (2021) screened for substitutions in AtKAI2 that could render it much more sensitive to the 2′*R*-configured enantiomer $GR24^{5DS}$. Using the 2′*R*-sensitive ShHTL7 from the parasitic weed *Striga hermonthica* as a guide, one AtKAI2 variant with three combined substitutions (Trp153Leu, Phe157Thr and Gly190Thr) showed the strongest responsiveness to $GR24^{5DS}$ in terms of Arabidopsis seed germination, without losing the capacity to respond to KAR_2, and potentially KL (Arellano-Saab *et al.*, 2021).

The recurring theme across these studies is one of *KAI2* gene duplication and subsequent diversification, which mirrors the independent recruitment of *KAI2* homologues in parasitic weeds for strigolactone perception (Conn *et al.*, 2015). Perhaps surprisingly, there are multiple solutions to altering KAI2 ligand specificity and affinity for karrikin-derived signals that appear to have evolved independently in different lineages.

Recognition of an activated receptor by MAX2

Although a MAX2-KAI2 complex has yet to be described, structural studies have revealed two modes for binding between MAX2/D3 and D14 that may also be relevant for KAI2. In the first, AtD14 was bound to the leucine-rich repeat (LRR) region of rice D3, with the covalently linked product of GR24 hydrolysis trapped in the ligand-binding pocket (Yao *et al.*, 2016). In the second, the extreme C-terminal LRR20 of D3 was captured in a complex with rice D14 in a pre-hydrolysis state (Shabek *et al.*, 2018). This C-terminal helix (CTH) of D3 is conformationally flexible and can adopt an engaged/closed form with a typical helical structure, or a dislodged/open state, with more of a random coil appearance. Interestingly, the CTH alone is able to bind and inhibit the hydrolysis activity of D14, suggesting that this region of D3 has a regulatory function (Shabek *et al.*, 2018). Both the engaged and dislodged forms have been observed in the two D3-D14 structures (Yao *et al.*, 2016; Shabek *et al.*, 2018), raising the question of whether CTH conformational flexibility has functional relevance in plants.

Recent data show that the C-terminal aspartate residue of MAX2, which is absolutely conserved, is important for the transition between the engaged and dislodged states (Tal *et al.*, 2022). In the engaged state, this negatively charged aspartate residue sits within a positively charged concave region on the surface called the D pocket. Mutation of the aspartate to lysine – which forces the CTH into the dislodged state – produces multiple phenotypes in Arabidopsis that are consistent with disrupted SL and KL signalling, as do mutations of the D pocket or CTH. Although polyubiquitination of a $D53_{D2}$ fragment is increased, proteasomal degradation of a $SMXL7_{D2}$ fragment is slowed by the Asp-to-Lys mutation. However, the capacity of MAX2/D3 to recruit the signalling partners D14 and D53/SMXLs is unaffected. This suggests that the dislodged CTH state is important for recruitment of a SMXL substrate, whereas the engaged state is important for proteasomal degradation of the substrate and signal transduction, putatively by promoting substrate release. Excitingly, Tal et al. (2022) also found that the switch between the two states could be affected by small organic acids with carboxylate groups that can fit in the D pocket. In particular, citrate promotes formation of the dislodged state of D3, thus rendering the $SMXL7_{D2}$ fragment more stable (Tal *et al.*, 2022). Therefore, allosteric control of MAX2 activity by metabolites may impose another layer of regulation for SMXL protein dynamics.

Degradation of SMXL repressor proteins

Genetic analysis and homology between KAR/KL and SL signalling components led to the long-held assumption that SMAX1 and SMXL2 are polyubiquitinated and targeted for degradation by KAI2-SCF^{MAX2} in a similar manner to D53-type SMXL degradation by D14-SCF^{MAX2} (Soundappan *et al.*, 2015; Waters *et al.*, 2017; Blázquez *et al.*, 2020). Until recently, however, biochemical evidence for this mechanism was lacking. The first demonstration of SMAX1 degradation showed that SMAX1-YFP expressed under the control of a *SMXL5* promoter in the developing phloem and procambium of the root disappears within minutes of *rac*-GR24 treatment (Wallner *et al.*, 2017). However, expression of *SMAX1-GFP* driven by a native *SMAX1* promoter did not produce detectable fusion protein, even in a *max2* background, although the transgene was functional (Khosla *et al.*, 2020a). Only deletion of a conserved RGKT motif, which also affects D53 stability (Jiang *et al.*, 2013; Zhou *et al.*, 2013), produced a stable, detectable fusion protein. Putatively, SMAX1 has a high rate of turnover that is not entirely due to MAX2 (Khosla *et al.*, 2020a). This problem was bypassed by adoption of a ratiometric reporter system and the use of a C-terminal SMAX1 fragment that has a longer half-life than full-length SMAX1. With these tools, it was shown that SMAX1 is degraded after karrikin or *rac*-GR24 treatment in a *KAI2*- and *MAX2*-dependent manner (Khosla *et al.*, 2020a). Recently, immunoblot detection of a 35S:SMAX1-GFP reporter in Arabidopsis seedlings was achieved, enabling another observation of KAR_2-induced degradation of SMAX1 (Park *et al.*, 2022). Better success has been had in demonstrating the polyubiquitination and degradation of Arabidopsis SMXL2 and rice OsSMAX1 after KAI2-SCF^{MAX2} activation (Wang *et al.*, 2020b; Zheng *et al.*, 2020). GFP-fused SMXL2 protein is degraded in Arabidopsis after KAR_1 and GR24$^{ent\text{-}5DS}$ treatment. OsSMAX1 abundance is increased in rice *d14l* or *d3* mutants (Choi *et al.*, 2020; Zheng *et al.*, 2020). OsSMAX1 also declines after treatment with KARs or GR24$^{ent\text{-}5DS}$ in a *D3*- and/or *D14L/KAI2*-dependent manner (Zheng *et al.*, 2020). Polyubiquitination of SMXL2 and OsSMAX1 is dependent on the GKT motif (Wang *et al.*, 2020b; Zheng *et al.*, 2020).

KAI2 interactions with SMAX1 and SMXL2 are enhanced by *rac*-GR24, in particular by the GR24$^{ent\text{-}5DS}$ component (Wang *et al.*, 2020b; Khosla *et al.*, 2020a). Interactions between ShHTL7, a paralog of KAI2 in *Striga hermonthica* that detects strigolactone, and MAX2 or SMAX1 are also respectively dependent on, or improved by, GR24 (Yao *et al.*, 2017). In some assays, however, AtKAI2 appears to be more prone to ligand-independent interactions with SMAX1 and MAX2 than D14 is with its partners (Xu *et al.*, 2018; Yao *et al.*, 2018; Khosla *et al.*, 2020a). Similarly, in several assays, OsD14L shows constitutive interactions with OsSMAX1 that are unaffected by the presence of an agonist or the loss of catalytic triad residues. Nor does OsD14L require an agonist to interact with D3 (Zheng *et al.*, 2020). Nonetheless, ligand-enhanced degradation of OsSMAX1 degradation shows that activation of OsD14L is still important for KAR/KL signalling in rice. It may be that KAI2/D14L proteins are “sticky” in many protein-protein interaction assays. Transient expression of *Lotus japonicus* KAI2a or KAI2b blocks the accumulation of detectable LjSMAX1-GFP reporter in *Nicotiana benthamiana* when LjMAX2 is coexpressed, and does not require application of a KAI2 agonist (Carbonnel *et al.*, 2020a). Perhaps LjKAI2a and LjKAI2b are activated by endogenous signals in *Nicotiana benthamiana*, or perhaps transient, ligand-independent interactions with LjMAX2 and LjSMAX1-GFP are sufficient to drive LjSMAX1-GFP degradation when LjMAX2 is overexpressed.

Despite the strong homology between the core components of KAR/KL and SL signalling, these pathways are mostly independent (Figure 3A). Loss of SMAX1 and SMXL2 suppresses *kai2*-associated phenotypes of *max2*, while loss of SMXL6,7,8 suppresses *d14*-associated phenotypes of *max2* (Soundappan *et al.*, 2015; Villaécija-Aguilar *et al.*, 2019). KAI2 does not interact with D53-type SMXL proteins or induce their degradation (Wang *et al.*, 2020b; Khosla *et al.*, 2020a; Carbonnel *et al.*, 2020a). Neither do karrikins inhibit shoot branching or trigger D53-type SMXL degradation (Nelson *et al.*, 2011; Jiang *et al.*, 2013; Wang *et al.*, 2015; Khosla *et al.*, 2020a; Song *et al.*, 2021). However, D14 is able to target SMAX1 and SMXL2 for degradation when GR24 is applied (Wang *et al.*, 2020b; Li *et al.*, 2022). This seems likely to be a non-preferred interaction that may not occur in many natural physiological contexts. For example, SL-insensitive and SL-deficient mutant seedlings do not show signs of SMAX1/SMXL2 overaccumulation, unlike *kai2* and *max2*, implying that endogenous strigolactones are not important for normal seedling photomorphogenic growth (Nelson *et al.*, 2011; Shen *et al.*, 2012). In addition, expression of *D14* under control of a *KAI2* promoter does not recover *kai2* seed germination, even in the presence of GR24 (Waters *et al.*, 2015b). This suggests that D14-mediated targeting of SMAX1 and SMXL2 is too inefficient to overcome seed dormancy. However, under osmotic stress, which putatively raises endogenous strigolactone levels, SMAX1 degradation occurs in a D14-SCF^{MAX2}-dependent manner (Li *et al.*, 2022).

Additional regulation of SMXL degradation

SMAX1 protein abundance is not only controlled by SCF^{MAX2}. SMAX1 shows instability *in vitro*, even in *max2* or *kai2* protein extracts, that is only partially slowed by treatment with the 26S proteasome inhibitor MG132 (Khosla *et al.*, 2020a). In one study, SMAX1-GFP could not be detected, even in a *max2* background (Khosla *et al.*, 2020a). SMAX1 abundance is markedly reduced by higher temperatures (e.g. 1 d at 28 C) (Park *et al.*, 2022). SMAX1-GFP protein levels in light-grown seedlings also decline several-fold within three hours of transfer to the dark (Kim *et al.*, 2022). This reduction is not blocked by MG132 treatment. This raises the possibility that environmental factors such as temperature and light can potentiate SCF^{MAX2}-mediated responses to KAR/KL by modulating SMAX1 abundance.

Nutrient availability or primary metabolites can tune SL signal transduction. D53 degradation is induced by nitrate and inhibited by sucrose (Sun *et al.*, 2021; Patil *et al.*, 2022). As discussed above, citrate allosterically influences conformational switches in MAX2 *in vitro* by blocking engagement of the CTH with the D pocket. This in turn impacts the polyubiquitination and degradation of D53 and SMXL7 (Tal *et al.*, 2022). KAR/KL signalling might be influenced similarly. In Arabidopsis, *max2* mutations that block CTH engagement produce elongated hypocotyls in seedlings (Tal *et al.*, 2022), which suggests disruption of KL perception and the possibility that SMAX1/SMXL2 degradation might be affected by an organic acid such as citrate. Another potential example is found in root chemotropism of the facultative parasite, *Phtheirospermum japonicum*. Growth of the parasite root toward strigolactones, which favours attachment to a host root, is putatively mediated by KAI2 proteins and only occurs under nitrogen deficiency (Ogawa *et al.*, 2022).

PHYTOCHROME-ASSOCIATED PROTEIN PHOSPHATASE 5 (PAPP5) is another potential regulator of MAX2 activity. PAPP5 was identified through affinity purification-mass

spectrometry as a potential interacting protein with MAX2, KAI2, and, to a lesser extent, D14 baits (Struk *et al.*, 2021). A surface-localised MAX2 phosphopeptide is detected in the *papp5* mutant but not in wild type, suggesting MAX2 is a potential substrate of PAPP5. However, *papp5* has weak phenotypes compared to *max2* and *kai2*. Seed dormancy in the dark and hypocotyl elongation are slightly enhanced in *papp5*, but shoot branching and lateral root growth remain normal (Struk *et al.*, 2021). Thus PAPP5 may have some effect on KAR/KL signalling. The functional importance of these candidate phosphorylation sites remains to be evaluated.

The functions of SMXL protein domains

SMAX1 and SMAX1-LIKE proteins are distantly related to the hexameric molecular chaperone, HEAT SHOCK PROTEIN 101 (HSP101) (Stanga *et al.*, 2013). SMXL proteins are composed of an N-terminal double Clp-N motif (N domain), a putative ATPase domain (D1), a middle region (M), and a C-terminal, putative ATPase domain (D2) that can be further subdivided into D2a and D2b (Figure 3B). The N domain is the most well-conserved region of SMXL proteins, but its function is currently unknown. It is not required for degradation of SMAX1 (Khosla *et al.*, 2020a; Park *et al.*, 2022). The D1 or D1M domains mediate interactions with D14 or KAI2 (Zhou *et al.*, 2013; Khosla *et al.*, 2020a; Zheng *et al.*, 2020). However, the D2 domain is also likely to contribute to interactions with the receptors and stabilise formation of tripartite SMXL-receptor-SCF^{MAX2} complexes (Shabek *et al.*, 2018). D2 is necessary for SCF^{MAX2}-induced degradation, but it is not sufficient because it lacks receptor interaction domains; thus it should not be termed a degron. $SMAX1_{D2}$ is degraded after GR24 treatment in wild-type Arabidopsis seedlings, but not in the *smax1 smxl2* background; it may be targeted for degradation indirectly through association with full-length SMAX1 or SMXL2, which have receptor-interaction domains (Khosla *et al.*, 2020a). A highly conserved Arg-Gly-Lys-Thr ("RGKT"), or P-loop, motif in the D2a domain is important for the stability of SMXL proteins. Deletion of the RGKT motif renders SMAX1, SMXL2, and D53-type SMXL proteins resistant to SCF^{MAX2}-mediated polyubiquitination and degradation, and causes dominant KAR/KL- or SL-insensitive effects (Jiang *et al.*, 2013; Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015, 2020b; Khosla *et al.*, 2020a; Zheng *et al.*, 2020; Carbonnel *et al.*, 2020a). SMXL3/4/5 proteins lack this motif and are not degraded after *rac*-GR24 treatment (Wallner *et al.*, 2017). The D2b domain is implicated in SMXL-SMXL protein-protein interactions and reduces the instability of SMAX1 variants that contain D2a (Khosla *et al.*, 2020a).

SMXL proteins are nuclear-localised. A nuclear localisation sequence is found in the D2a domain of SMAX1 and in the N domain of SMXL7 (Liang *et al.*, 2016; Khosla *et al.*, 2020a). MAX2 is also nuclear-localised, while KAI2 and D14 are found in both the nucleus and cytoplasm (Stirnberg *et al.*, 2007; Shen *et al.*, 2007; Sun & Ni, 2011; Chevalier *et al.*, 2014; Liang *et al.*, 2016). This implies that the nucleus is the site of SMXL protein function and post-translational regulation. Supporting this idea, nuclear localisation is important for the function and SL-induced degradation of SMXL7 (Liang *et al.*, 2016).

SMXL proteins share a conserved Ethylene-responsive element binding factor-associated Amphiphilic Repression (EAR) motif in D2a that enables interactions with TOPLESS and TOPLESS-RELATED proteins, implying that they function as transcriptional co-repressors (Jiang *et al.*, 2013; Soundappan *et al.*, 2015; Wang *et al.*, 2015; Struk *et al.*, 2018; Zheng *et al.*, 2020). The EAR motif has varying importance for different SMXL7-regulated developmental processes (Liang *et al.*, 2016). D53 also has a second, non-conserved EAR

motif that contributes to some of its functions (Ma *et al.*, 2017). It has been assumed that SMXL proteins recruit TPL/TPR to genomic loci that are specified through interactions with transcription factors, analogous to the Aux/IAA proteins of auxin signalling and the JAZ-NINJA complex of jasmonate signalling (Blázquez *et al.*, 2020). Indeed, D53 proteins in rice, wheat, maize, and Arabidopsis interact with SQUAMOSA-PROMOTER BINDING PROTEIN-LIKE (SPL) proteins to regulate shoot branching (Liu *et al.*, 2017, 2021; Song *et al.*, 2017; Xie *et al.*, 2020; Sun *et al.*, 2021). The transcription factors BES1 in Arabidopsis and BZR1 in rice serve as additional regulators of shoot branching through association with D53 (Fang *et al.*, 2020; Hu *et al.*, 2020). Unexpectedly, D53 orthologs in Arabidopsis also show the ability to bind DNA directly (Wang *et al.*, 2020a). The "output" domain(s) of SMXL proteins that determine specificity in DNA-binding or interactions with DNA-binding proteins have not yet been resolved. Transcription factor partners of SMAX1 and SMXL2 have also not yet been identified, although SPL proteins may be a reasonable starting point for candidates.

A recent review has suggested an additional role for the EAR motif with the intriguing hypothesis that SMXL proteins form molecular condensates that influence their function (Temmerman *et al.*, 2022). This idea is based upon the observation that SMXL translational reporters can form subnuclear speckles, either on their own or when in association with TPR2 or D14 (Zhou *et al.*, 2013; Soundappan *et al.*, 2015; Liang *et al.*, 2016). Putatively, these condensates could form through a chain of associations between the EAR motifs in SMXL-SMXL complexes and tetrameric TPL/TPR proteins (Ma *et al.*, 2017; Temmerman *et al.*, 2022). One simple test will be to determine whether the formation of nuclear speckles by SMXL proteins is EAR motif-dependent.

Events further downstream that link SMAX1/SMXL2 degradation to specific developmental outputs are gradually emerging and complex. For brevity, we will only note here that KAR/KL signalling integrates with light, temperature, auxin, ethylene, abscisic acid, brassinosteroid, and gibberellin pathways (Nelson *et al.*, 2009, 2010; Waters & Smith, 2013; Wei *et al.*, 2016; Brun *et al.*, 2019; Bunsick *et al.*, 2020, 2022; Carbonnel *et al.*, 2020a; Bunsick & Lumba, 2021; Bursch *et al.*, 2021; Villaécija-Aguilar *et al.*, 2022; Hamon-Josse *et al.*, 2022; Park *et al.*, 2022; Kim *et al.*, 2022). This will be a rich area for future study.

Feedback regulation of KAR/KL signalling

At least three mechanisms contribute to negative feedback regulation of KAR/KL signalling, which is likely important for limiting the duration and intensity of KAR/KL responses. First, KAI2 protein is subject to degradation within hours of treatment with KAR_2, $GR24^{ent\text{-}5DS}$, or $dGR24^{ent\text{-}5DS}$, limiting the amount of receptor available for continued perception (Waters *et al.*, 2015a; Yao *et al.*, 2021). This occurs through interaction with SMAX1 and SMXL2 but, unlike D14, is not dependent on SCF^{MAX2} (Chevalier *et al.*, 2014; Khosla *et al.*, 2020a). Second, *SMAX1* and/or *SMXL2* transcripts increase after karrikin or *rac*-GR24 treatment, and are downregulated in *max2* and rice *d14l* (Mashiguchi *et al.*, 2009; Nelson *et al.*, 2010; Stanga *et al.*, 2013; Zheng *et al.*, 2020). Putatively, this tunes the amount of KAI2-SCF^{MAX2} substrate that must be degraded for a response to occur.

Third, a recent characterization of a transcriptional marker of KAR/KL signalling, *KARRIKIN UPREGULATED F-BOX1* (*KUF1*), in Arabidopsis fortuitously revealed a gene that appears to negatively regulate KL biosynthesis and KAR metabolism (Sepulveda *et al.*, 2022). The loss-

of-function *kuf1* mutant shows several phenotypes that are consistent with hyperactive KAR/KL signalling and dependent on *MAX2* and *KAI2* (Sepulveda *et al.*, 2022; Feng *et al.*, 2022) (Tian et al., *Plant Physiology*, accepted 6/26/22). Intriguingly, *kuf1* shows hypersensitivity to KAR_1 but not other signals mediated by KAI2 such as KAR_2 or GR24, which can be perceived directly. This suggests that *kuf1* is affected in a step upstream of ligand perception by KAI2, rather than having a defect that affects the activity of the KAR/KL signalling pathway overall (Sepulveda *et al.*, 2022). As an F-box protein, KUF1 is likely to target one or more protein substrates for ubiquitination and degradation. This target(s) may directly or indirectly promote KL biosynthesis and KAR metabolism.

Another implication of these observations with *kuf1* is that KAR_1 and KAR_2 are not metabolised by the same protein in Arabidopsis. Some potential support for this idea comes from the observation that *Lotus japonicus* has tissue-specific responses to KAR_1 and KAR_2 (Carbonnel *et al.*, 2020b). LjKAI2a confers responses to KAR_1 and KAR_2 in *L. japonicus* hypocotyls and when expressed in Arabidopsis. However, *L. japonicus* roots respond to KAR_1 alone, despite *LjKAI2a* being expressed and active. This suggests that another factor needed for KAR_2 perception, perhaps a KAR_2-metabolising enzyme, is not expressed in roots.

It may be that other genes regulated by KAR/KL participate in feedback regulation loops. For example, *DLK2* expression is upregulated in response to karrikins or removal of SMAX1 and SMXL2, and downregulated in *max2* and *kai2* (Waters *et al.*, 2012; Stanga *et al.*, 2013, 2016). Interestingly, *DLK2* expression is also increased in response to root colonisation by AM fungi in tomato and rice (Ho-Plágaro *et al.*, 2021; Sisaphaithong *et al.*, 2021). The function of *DLK2* with regard to SCF^{MAX2}-dependent signalling is currently unclear (Végh *et al.*, 2017), but an Arabidopsis *dlk2* mutant has shown reduced seed dormancy under light-limited conditions, which could be consistent with increased KAR/KL signalling (Bunsick *et al.*, 2022). In tomato, *DLK2* overexpression in roots reduces AM colonisation and inhibits arbuscule branching, whereas *DLK2* silencing has the opposite effect. The proposed explanation for this is that DLK2 sequesters DELLA proteins by protein-protein interaction, inhibiting the promotion of arbuscule development by DELLAs (Ho-Plágaro *et al.*, 2021). Alternatively, given that AM colonisation is a *KAI2/D14L*-dependent process in rice and *Brachypodium distachyon* (Gutjahr *et al.*, 2015; Choi *et al.*, 2020; Meng *et al.*, 2022), it is possible that *DLK2* inhibits some KAR/KL responses.

Conclusion

The smoke has cleared substantially over the past 18 years since the discovery of karrikins, leaving us with a far better understanding of how karrikin and KL signalling affects plant growth. However, many burning questions remain. What are the identities of KL and the putative karrikin metabolites? How is KL made and how are karrikins metabolised? Is KL one compound or many, and is it the same compound across the green lineage? How are KAR/KL and strigolactone signalling pathways insulated from each other, and how did this separation evolve? How are different developmental responses controlled by KAR/KL signalling? We anticipate that the answers to these questions will spark future innovations in agriculture, while illuminating a fascinating phenomenon in plant biology.

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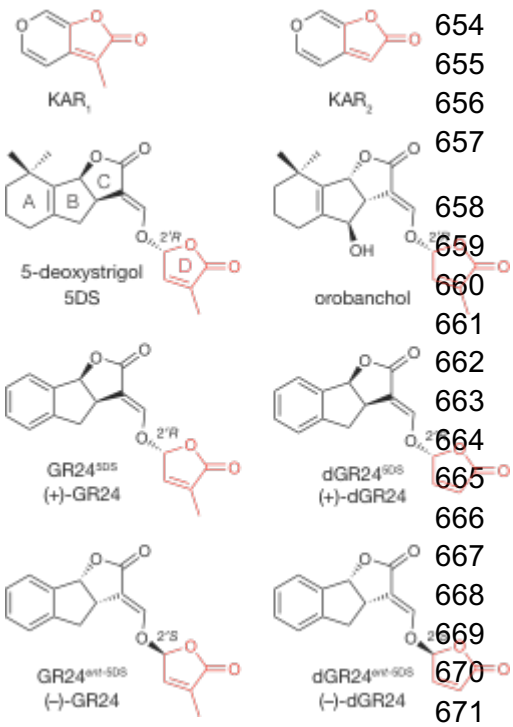
BOXES

BOX 1 - Bioactive butenolides



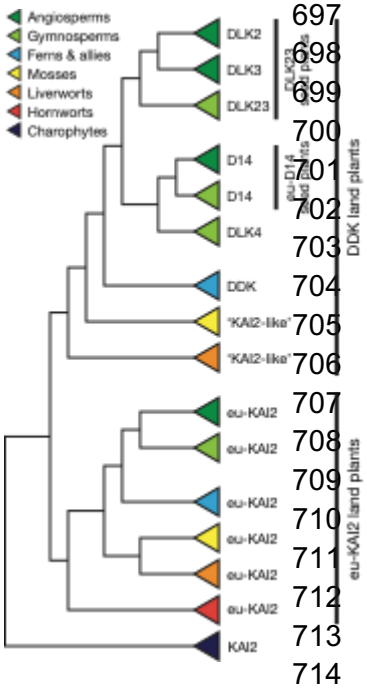
Karrikins are chemically classed as butenolides based on the four-carbon, heterocyclic lactone structure. A number of other bioactive butenolides, both natural and synthetic, have been described, most notably strigolactones (SLs).

Karrikins and strigolactones share a butenolide moiety (red) that is essential for bioactivity. KAR_1 and KAR_2 are the most frequently used karrikins in recent literature, and differ only by a methyl group. KAR_2 is more potent than KAR_1 in Arabidopsis, but karrikin preferences can vary for different species or traits (Flematti *et al.*, 2007; Nelson *et al.*, 2009, 2010; Sun *et al.*, 2020; Carbonnel *et al.*, 2020b; Martinez *et al.*, 2022). Canonical strigolactones have a tricyclic lactone core (the ABC part) linked by an enol-ether bridge to a butenolide moiety, also known as the D-ring. Non-canonical SLs, which are also derived from β-carotene via a carlactone intermediate, lack the ABC-rings of canonical SLs but share the enol-ether-linked D-ring (Yoneyama *et al.*, 2018). Similarly, synthetic SL substitutes and profluorescent SL probes such as Yoshimulactone Green, Xilatone Red, and the GC series commonly feature an ether-linked methyl butenolide (Tsuchiya *et al.*, 2015; Wang *et al.*, 2021b; de Saint Germain *et al.*, 2022). This underscores the significance of the D-ring for receptor activation, whereas other parts of the molecule can vary substantially.

Canonical SLs are split into two main groups – represented here by 5-deoxystrigol and orobanchol – as defined by the stereochemistry of the B-C junction. Although this stereochemistry can influence the germination of parasitic weeds, it is not a critical determinant of bioactivity in the control of shoot branching via D14 (Fukui *et al.*, 2011; Zwanenburg & Pospísil, 2013; Boyer *et al.*, 2014; Nelson, 2021). In contrast, the 2′ carbon of the D-ring is exclusively in the *R* configuration in naturally occurring strigolactones. This stereochemistry originates in the strigolactone precursor carlactone (Alder *et al.*, 2012). However, the opposite D-ring configuration (2′*S*) is produced during synthetic preparations of strigolactone analogues such as GR24. Molecules with a 2′*S*-configured D-ring are much less active via the strigolactone receptor D14 compared to the natural 2′*R* configuration (see main text). The two enantiomers can either be separated by chiral-phase HPLC (Scaffidi *et al.*, 2014) or synthesised directly by enantioselective techniques (Bromhead *et al.*, 2014). Typical preparations of GR24 are racemic mixtures (*rac*-GR24) of both 2′*R* and 2′*S* enantiomers. These enantiomers are commonly referred to as either (+)-GR24 and (–)-GR24, or $GR24^{5DS}$ and $GR24^{ent\text{-}5DS}$, respectively. Also shown here are “desmethyl” equivalents of the GR24 enantiomers, which lack the butenolide methyl group, similar to KAR_2. These desmethyl

694 compounds are seemingly not bioactive via D14, but dGR24$^{ent\text{-}5DS}$ is particularly active via
695 KAI2 orthologues from numerous species (Yao *et al.*, 2021).

BOX 2 - Evolution of the KAI2 and D14 family of α/β-hydrolases

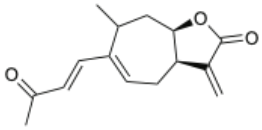


The KAI2/D14 family may have emerged in land plants via horizontal gene transfer from bacteria (Wang *et al.*, 2022). Available evidence suggests an origin for the family in the Charophyte algae, followed by an ancient split very early in the evolution of land plants to form two super-clades: *eu-KAI2* and *DDK* (for *D14*/*DLK2*/*KAI2*), as depicted in this simplified phylogeny. The *eu-KAI2* clade is generally highly conserved, and contains the characterised *KAI2* sequences from angiosperms, along with highly similar sequences from gymnosperms, pteridophytes and lycophytes (here collapsed into "Ferns & allies"), mosses, liverworts and hornworts. Thus, all land plant groups are represented in this clade. The *DDK* clade is much more divergent on a sequence level. It contains previously characterised *D14* and *DLK2* homologues from angiosperms, but also one or more 'KAI2-like' sequences from mosses (*PpKAI2L-FK, -HIL* and *-GJM*) (Lopez-Obando *et al.*, 2021), liverworts (*MpKAI2b*) (Mizuno *et al.*, 2021), and *Selaginella* (*SmKAI2b*) (Waters *et al.*, 2015b) that had not resolved clearly with core *KAI2* or *D14* sequences in previous analyses (Waters *et al.*, 2012; Lopez-Obando *et al.*, 2016). In reality, many of these 'KAI2-like' *DDK* members are no more similar to *eu-KAI2* than they are to *eu-D14* (Bythell-Douglas *et al.*, 2017). The conserved *D14* sequences, restricted to seed plants, are defined as *eu-D14* to distinguish them from uncharacterised close relatives in the *DLK4* clade found in gymnosperms. Taxon-specific expansions within the *DDK* clade have given rise to additional groups such as *DLK3* (close relatives of *DLK2* in angiosperms) and *DLK23* (a gymnosperm-specific sister group of *DLK2* and *DLK3*). Perhaps surprisingly, the *DDK* superclade does not have any hornwort representatives. In addition, the precise placing of the hornwort members of the *eu-KAI2* super-clade is problematic, which casts some uncertainty about the timing of the super-clade split and therefore the origin of strigolactone perception within the *DDK* lineage (Bythell-Douglas *et al.*, 2017). This ambiguity results largely from the unresolved phylogenetic relationships of non-vascular plants.

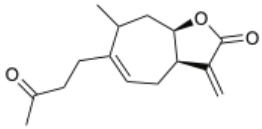
BOX 3 - Evidence that karrikins are not directly perceived by KAI2

Several observations demonstrate that KAR_1 does not activate KAI2 directly. First, in DSF assays, the melting temperature of KAI2 decreases in the presence of $GR24^{ent\text{-}5DS}$, $dGR24^{ent\text{-}5DS}$, and other 2′*S*-configured strigolactone analogues, but is unaffected by KAR_1 or KAR_2 (Waters *et al.*, 2015b; Yao *et al.*, 2018, 2021; Sun *et al.*, 2020). Second, yeast two-hybrid interactions between KAI2 and SMAX1 are stimulated by *rac*-GR24 and $GR24^{ent\text{-}5DS}$, but not by KAR_1, KAR_2, or the 2′*R*-configured SL analog $GR24^{5DS}$ (Khosla *et al.*, 2020a). Third, co-immunoprecipitation of KAI2 and SMXL2 occurs in the presence of $GR24^{ent\text{-}5DS}$, but not KAR_1 (Wang *et al.*, 2020b). Likewise, *rac*-GR24 stimulates KAI2-dependent interaction with MAX2 in pull-down assays, but KAR_1 does not (Xu *et al.*, 2018). Fourth, stimulation of SMXL2 polyubiquitination and degradation *in vivo* is slower with KAR_1 than with $GR24^{ent\text{-}5DS}$ (Wang *et al.*, 2020b). Similarly, OsSMAX1 degradation in rice calli is apparent within 30 min of treatment with $GR24^{ent\text{-}5DS}$, but KAR_1 has no effect even after a 2 h treatment (Zheng *et al.*, 2020). Fifth, crystallography of two KAI2-KAR_1 complexes are inconsistent with respect to KAR_1 orientation in the ligand-binding pocket (Guo *et al.*, 2013; Xu *et al.*, 2016). Neither structure shows the dramatic conformational change found in D14 when it is in a putatively active complex with MAX2/D3 (Yao *et al.*, 2016). Finally, the chemistry of karrikins is incompatible with the ligand hydrolysis model proposed for PsKAI2B in pea (Guercio *et al.*, 2022), because karrikins do not have a suitable leaving group and would likely re-close upon nucleophilic attack (Scaffidi *et al.*, 2012). However, this last point may be moot if ligand hydrolysis is not essential for signal transduction by KAI2, as has been hypothesised for D14 (Seto *et al.*, 2019).

BOX 4 - What is the endogenous KAI2 ligand?



8-epixanthatin



tomentosin

Although the identity of the KAI2 ligand(s) (KL) remains mysterious, there are several clues. Putatively KL-responsive KAI2 proteins can be activated by synthetic molecules with hydrolyzable, 2'*S*-configured butenolide rings, such as GR24$^{ent\text{-}5DS}$ (Waters *et al.*, 2015b; Sun *et al.*, 2020; Wang *et al.*, 2020b; Carbonnel *et al.*, 2020b; Yao *et al.*, 2021). The KAI2 catalytic triad undergoes a similar modification with the cleaved butenolide ring as D14 does during strigolactone hydrolysis (Guercio *et al.*, 2022). This suggests KL may have some structural similarity to strigolactones. However, KL is unlikely to be derived from carlactone as strigolactones are. Carlactone-deficient mutants in the strigolactone biosynthetic pathway do not show *kai2* phenotypes, and the effects of carlactone application are dependent on D14 rather than KAI2 (Nelson *et al.*, 2011; Scaffidi *et al.*, 2013). The sesquiterpene lactones 8-epixanthatin and tomentosin, which have an unsaturated lactone moiety similar to a butenolide ring, have been proposed as candidate KAI2 ligands. This hypothesis is based on the observation that sesquiterpene lactones can inhibit hypocotyl elongation, are predicted to have high affinity to KAI2 structural models in molecular docking studies, and are potentially widespread in land plants as sesquiterpene derivatives (Rahimi & Bouwmeester, 2021). However, there is currently no experimental evidence that sesquiterpene lactones act via KAI2. Another idea is that KL may be a desmethyl butenolide because desmethyl, 2' epimer versions of strigolactone analogues (e.g. dGR24, Box 1) are strong and specific activators of KAI2 from a range of species (Yao *et al.*, 2021). However, in the absence of any KL-deficient mutants, the potential biosynthetic source of a desmethyl compound is unknown.

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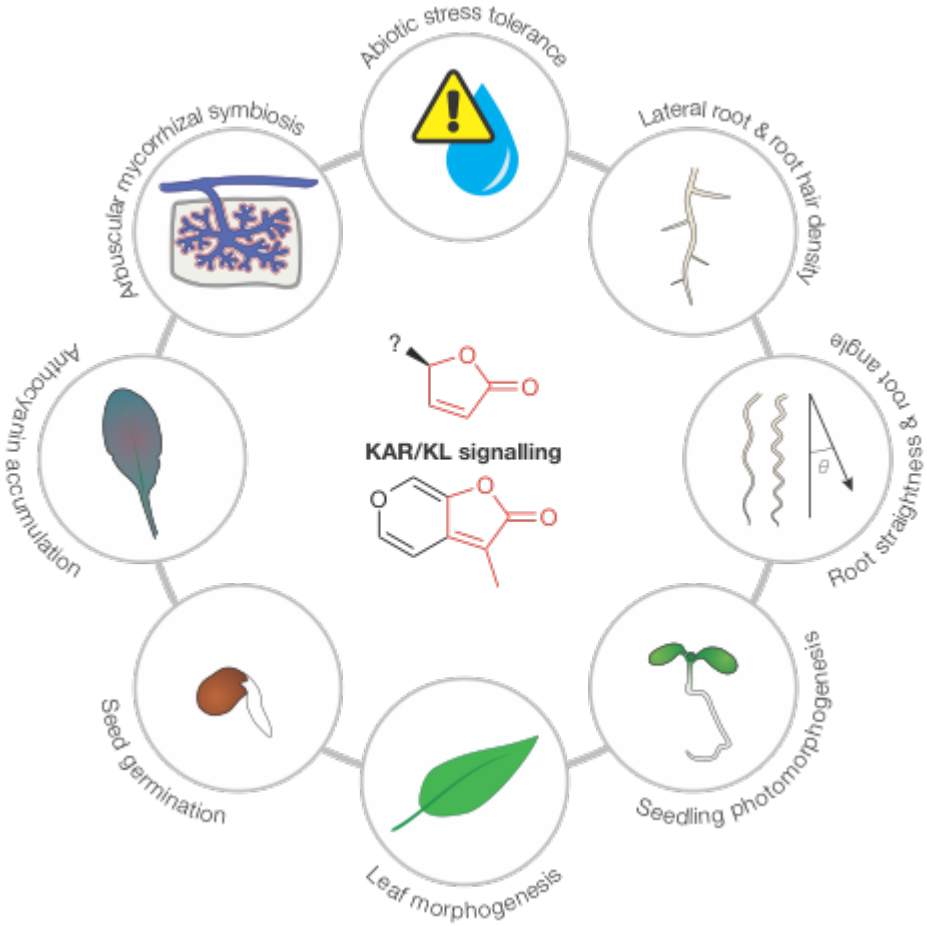

Figure 1. Roles of karrikin/KL signalling in plant development.

A summary of the broad developmental processes under the influence of KAR/KL signalling. These roles are supported by genetic analysis in combination with appropriate chemical treatments. Here KL is depicted, speculatively, as a desmethyl butenolide group with an unknown substituent moiety "?".

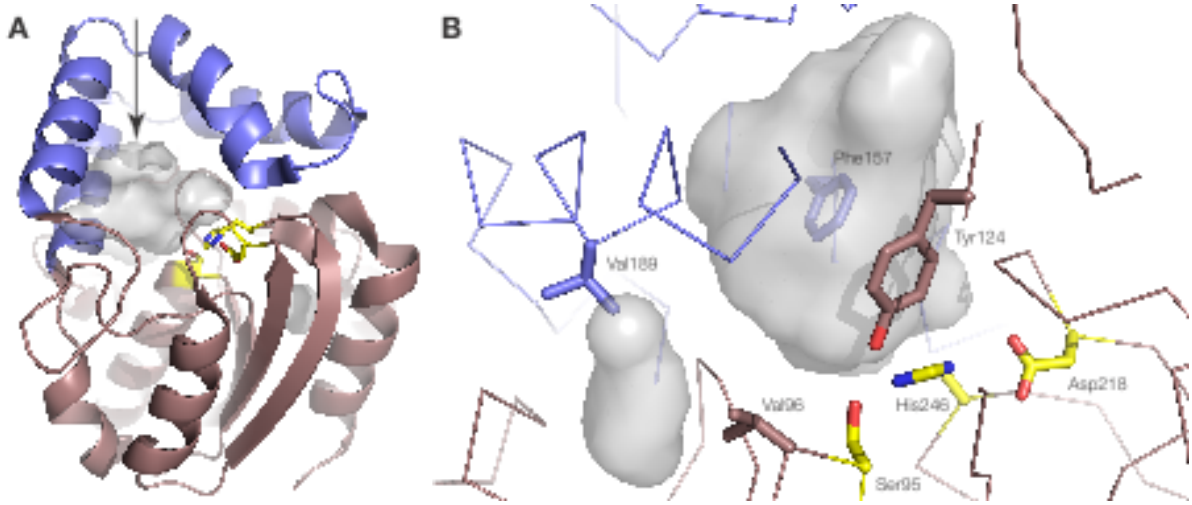

Figure 2. Key residues conferring ligand selectivity in KARRIKIN INSENSITIVE 2.

A. Overall structure of KAI2 from *Arabidopsis thaliana* (PDB 5Z9G; Lee *et al*. (2018)) highlighting the two V-shaped pairs of alpha helices that comprise the cap domain (blue) and the alpha-beta fold core domain (brown). Also shown are the catalytic Ser, His and Asp residues (yellow sticks) at the distal end of a ligand-binding pocket (grey surface, centre). Arrow indicates point of ligand entry from the solvent.

B. Closer view of the ligand-binding pocket of *Arabidopsis thaliana* KAI2. Sites that differ in KAI2 proteins from other species and that confer altered ligand specificity for KAR_1 versus KAR_2 are shown, coloured by domain. The conserved catalytic residues are shown as a reference point (yellow). Residue numbering is for AtKAI2.

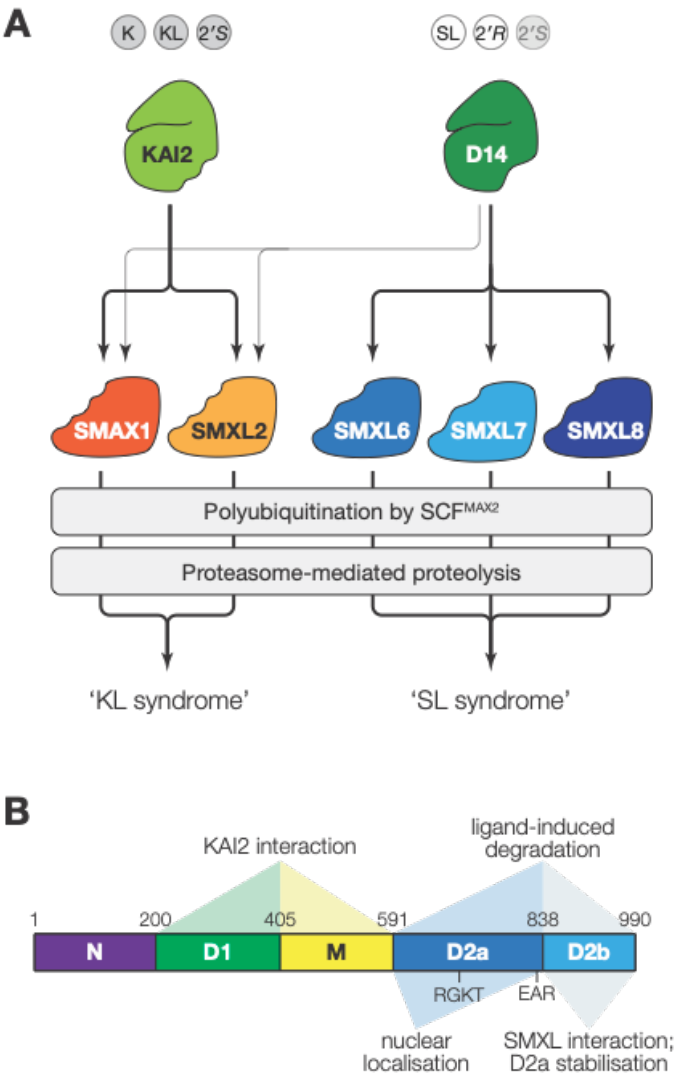

Figure 3. Regulation and domain structure of SMXL proteins.

A. Model of MAX2-dependent signalling in Arabidopsis. Upon activation by a putative karrikin-derived molecule (K), endogenous KAI2 ligand (KL), or butenolide molecules with a 2'*S* stereochemical configuration (e.g. GR24$^{ent\text{-}5DS}$ or dGR24$^{ent\text{-}5DS}$), the a/b-hydrolase KAI2 works with SCF^{MAX2} to target SMAX1 and SMXL2 proteins for polyubiquitination and proteasomal degradation. Similarly, D14 works with SCF^{MAX2} to target the D53-type SMXL proteins SMXL6, SMXL7, and SMXL8. However, D14 is activated by strigolactones (SL), butenolide molecules in a 2'*R* stereochemical configuration (e.g. GR24^{5DS}), and to a lesser degree by butenolide molecules in a 2'S configuration (e.g. GR24$^{ent\text{-}5DS}$). D14 can also target SMAX1 and SMXL2, when adequate agonist is present. Degradation of SMXL proteins leads to different downstream developmental responses.

B. Diagram of the major domains of Arabidopsis SMAX1 and their functions. Adapted from (Khosla et al., 2020). Other SMXL proteins have a similar overall structure. However, SMXL3, SMXL4, and SMXL5 lack the RGKT motif and the function of their domains has not been evaluated. The nuclear localisation sequence is not necessarily found in the D2a domain in D53-type SMXL proteins.

Table 1. Ligand preferences of homologues of KARRIKIN INSENSITIVE2

KAI2 homologues listed here are limited to those for which karrikin response has been investigated directly by plant-based assays and mutants, or by using heterologous complementation assays in *Arabidopsis thaliana*. Many *KAI2* homologues in parasitic plants that function as strigolactone receptors have also been examined through biochemical or transgenic approaches but are not listed here (see Nelson et al., 2021 for review). n.d., not determined; N/A, not applicable

Species	Homologue	Karrikin (KAR) preference	Other preferred ligands	Notes	Reference
Dicots					
Arabidopsis thaliana	AtKAI2	KAR_2>KAR_1	dGR24$^{ent\text{-}5DS}$	Crystal structure with KAR_1 reported	(Nelson *et al.*, 2009; Waters *et al.*, 2012; Guo *et al.*, 2013; Yao *et al.*, 2021)
Brassica tournefortii	BtKAI2a	KAR_2>KAR_1		No response to GR24 in DSF	(Sun *et al.*, 2020)
	BtKAI2b	KAR_1>KAR_2	GR24$^{ent\text{-}5DS}$	Dominant isoform in seeds and seedlings	
	BtKAI2c	N/A	N/A	Non-functional due to mutation	
Lactuca sativa	LsKAI2a	weak response to KAR			(Martinez *et al.*, 2022)
	LsKAI2b	KAR_1>KAR_2			
Pisum sativum	PsKAI2A	Ambiguous		Uncertain contribution of PsKAI2A to KAR response in *P. sativum*	(Guercio *et al.*, 2022)
	PsKAI2B	KAR_1>KAR_2 (probable)	GR24$^{ent\text{-}5DS}$	May not confer KL response in *A. thaliana*	
Lotus japonicus	LjKAI2a	KAR_1=KAR_2	GR24$^{ent\text{-}5DS}$		(Carbonnel *et al.*, 2020b)
	LjKAI2b	KAR_1>KAR_2		No response to GR24 in DSF	
Striga hermonthica	ShHTL3 (ShKAI2iB)	KAR_1=KAR_2		Crystal structure of ShHTL3 with KAR_1 reported; no activity with *rac*-GR24	(Conn *et al.*, 2015; Toh *et al.*, 2015; Xu *et al.*, 2016)
Phelipanche ramosa	PrKAI2d3	n.d.	GR24^{5DS}	Binds isothiocyanates *in vitro*	(de Saint Germain *et al.*, 2021)
Monocots					
Brachypodium distachyon	BdKAI2	KAR_2>KAR_1	dGR24$^{ent\text{-}5DS}$		(Meng *et al.*, 2022)

Oryza sativa	OsKAI2 (D14L)	Ambiguous	dGR24$^{ent\text{-}5DS}$	SMAX1 degradation response similar for KAR_1 and KAR_2	(Zheng *et al.*, 2020; Yao *et al.*, 2021)
Non-angiosperms					
Selaginella moellenorffii	SmKAI2a	No response to KAR	dGR24$^{ent\text{-}5DS}$	Retains KL response when expressed in *A. thaliana*	(Waters *et al.*, 2015b; Yao *et al.*, 2021)
Physcomitrium patens	PpKAI2-like (multiple)	No response to KAR	Generally GR24$^{ent\text{-}5DS}$	Extensive gene duplication in *P. patens*; no response to KAR in moss or in *A. thaliana* transgenics, but binding to KAR_1 reported for PpKAI2L-H, -K and -L	(Hoffmann *et al.*, 2014; Bürger *et al.*, 2019; Lopez-Obando *et al.*, 2021)
Marchantia polymorpha	MpKAI2a	No response to KAR	GR24$^{ent\text{-}5DS}$	Required for thallus growth and orientation; likely KL receptor	(Mizuno *et al.*, 2021)
	MpKAI2b	No response to KAR	GR24$^{ent\text{-}5DS}$	No clear developmental role	
Marchantia paleacea	MpaKAI2a	n.d.	GR24$^{ent\text{-}5DS}$	Required for thallus growth and orientation; likely KL receptor	(Kodama *et al.*, 2022)
	MpaKAI2b	n.d.		No clear developmental role	

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