The strigolactone receptor D14 targets SMAX1 for degradation in response to GR24 treatment and osmotic stress

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https://doi.org/10.1016/j.xplc.2022.100303

ABSTRACT

The effects of the phytohormone strigolactone (SL) and smoke-derived karrikins (KARs) on plants are generally distinct, despite the fact that they are perceived through very similar mechanisms. The homologous receptors DWARF14 (D14) and KARRIKIN-INSENSITIVE2 (KAI2), together with the F-box protein MORE AXILLARY GROWTH2 (MAX2), mediate SL and KAR responses, respectively, by targeting different SMAX1-LIKE (SMXL) family proteins for degradation. These mechanisms are putatively well-insulated, with D14-MAX2 targeting SMXL6, SMXL7, and SMXL8 and KAI2-MAX2 targeting SMAX1 and SMXL2 in Arabidopsis thaliana. Recent evidence challenges this model. We investigated whether D14 can target SMAX1 and whether this occurs naturally. Genetic analysis indicates that the SL analog GR24 promotes D14-SMAX1 crosstalk. Although D14 shows weaker interactions with SMAX1 than with SMXL2 or SMXL7, D14 mediates GR24-induced degradation of SMAX1 in plants. Osmotic stress triggers SMAX1 degradation, which is protective, through SL biosynthesis and signaling genes. Thus, D14-SMAX1 crosstalk may be beneficial and not simply a vestige of the evolution of the SL pathway.

Key words: phytohormone, signaling, proteolysis, crosstalk

Li Q., Martín-Fontecha E.S., Khosla A., White A.R.F., Chang S., Cubas P., and Nelson D.C. (2022). The strigolactone receptor D14 targets SMAX1 for degradation in response to GR24 treatment and osmotic stress. Plant Comm. 3, 100303.

INTRODUCTION

Strigolactones (SLs) and karrikins (KARs) are two classes of butenolide molecules that regulate diverse aspects of plant development. SLs were discovered in root exudates as germination stimulants of root-parasitic plants (Cook et al., 1966; Bouwmeester et al., 2021). SLs exuded into soil promote symbiotic interactions between roots and arbuscular mycorrhizal fungi, partly by stimulating hyphal branching (Akiyama et al., 2005; Gomez-Roldan et al., 2008; Kobae et al., 2018). SLs are also plant hormones with many roles, including the regulation of shoot branching, root growth, cambial growth, senescence, defense, and anthocyanin biosynthesis (Gomez-Roldan et al., 2008; Umehara et al., 2008; Agusti et al., 2011; Rasmussen et al., 2012; Van Ha et al., 2014; Yamada et al., 2014; Soundappan et al., 2015; Ueda and Kusaba, 2015; Lahari et al., 2019; Nasir et al., 2019; Villaécija-Aguilar et al., 2019; Wang et al., 2020a; Li et al., 2020b; Kalliola et al., 2020). KARs are abiotic signals found in smoke and biochar (Fiematti et al., 2004; Kochanek et al., 2016). They promote germination of many plant species after fire but can also stimulate species from non-fire-prone environments such as Arabidopsis thaliana (Fiematti et al., 2004; Nelson et al., 2012). In addition, KAR signaling influences seedling photomorphogenesis, mesocotyl elongation, root and root hair growth, and abiotic stress responses (Jain et al., 2006; Nelson et al., 2010; Li et al., 2017, 2020b; Wang et al., 2018; Swarbreck et al., 2019; Villaécija-Aguilar et al., 2019; Zheng et al., 2020).

Despite their different sources and effects, SLs and KARs are perceived similarly (Blázquez et al., 2020). The core SL signaling pathway in angiosperms consists of the receptor DWARF14 (D14)/DECREASED APICAL DOMINANCE2 (DAD2)/RAMOSUS3 (RMS3), the F-box protein DWARF3 (D3)/MORE AXILLARY GROWTH2 (MAX2), and transcriptional co-repressors in the...
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Umehara et al., 2008; Hamiaux et al., 2012; Waters et al., 2012; Jiang et al., 2013; Stanga et al., 2013; Zhou et al., 2013; de Saint Germain et al., 2016). D14 is an α/β-hydrolase that cleaves an enol-ether-linked methylbutenolide “D-ring” from SLs (Hamiaux et al., 2012; Seto et al., 2019). The D-ring becomes covalently attached to a His residue in the catalytic triad (de Saint Germain et al., 2016; Yao et al., 2016). D14 changes conformation during SL binding or hydrolysis, promoting interactions with D3/MAX2 and D53/SMXL6/7/8 (Jiang et al., 2013; Zhou et al., 2013; Wang et al., 2015; Yao et al., 2016). D14 is central to the formation of the tripartite complex, but D3 and D53 help stabilize the complex (Jiang et al., 2016; Shabek et al., 2018). D3/MAX2 functions within an Skp1, Cullin, F-box (SCF)-type E3 ubiquitin ligase complex. SCF^MAX2 polyubiquitinates D3/SMXL6/7/8 proteins, which are then rapidly degraded by the 26S proteasome (Jiang et al., 2013; Zhou et al., 2013; Soundappan et al., 2015; Wang et al., 2015; Yao et al., 2016; Shabek et al., 2018). D14 is also degraded after SL activation in a MAX2-dependent manner, but this occurs over hours rather than minutes (Chevalier et al., 2014; Hu et al., 2017).

KAR signaling shares a requirement for MAX2, but the ancient D14 paralog KARRIKIN INSENSITIVE 2 (KAI2)/HYPOSENSITIVE TO LIGHT (HTL) acts as a receptor, and SMAX1 and SMXL2 are downstream targets (Nelson et al., 2011; Sun and Ni, 2011; Waters et al., 2012; Stanga et al., 2013, 2016; Khosla et al., 2020b; Zheng et al., 2020). Similar to SL signaling, the activation of KAI2 triggers its association with MAX2 and SMAX1/SMXL2, leading to SMAX1 and SMXL2 degradation (Yao et al., 2017; Xu et al., 2018; Carbonnel et al., 2020a; Khosla et al., 2020b; Wang et al., 2020b, 2021; Zheng et al., 2020). Polyubiquitination has been demonstrated for SMXL2 and is presumed for SMAX1 (Wang et al., 2020b). KAI2 is also degraded after activation, although unlike D14, this is SMAX1/SMXL2-dependent rather than MAX2-dependent (Waters et al., 2015b; Khosla et al., 2020b). In addition to mediating KAR responses, KAI2 is thought to recognize an endogenous signal, KAI2 ligand (KL), that remains undiscovered (Waters et al., 2015a; Conn and Nelson, 2015). KAI2 is more sensitive to desmethyl butenolide compounds than methylbutenolide compounds, which may give hints about the chemical structure of KL (Yao et al., 2021). KARs themselves are likely to require metabolism in plants for recognition by KAI2 (Waters et al., 2015a; Xu et al., 2018; Khosla et al., 2020b; Wang et al., 2020b; Nelson, 2021).

There is substantial evidence that SL and KAR/KL pathways function independently despite their homology. First, SL and KAR treatments usually affect different aspects of plant growth (Waters et al., 2017). For example, SLs inhibit shoot branching, whereas KARs promote Arabidopsis germination (Nelson et al., 2011; Scaffidi et al., 2014). Second, genetic analysis often shows different roles for SL and KAR/KL pathway genes. SL-insensitive and SL-deficient mutants often have different phenotypes than the KAR/KL-insensitive mutant kai2 (Nelson et al., 2011; Waters et al., 2012; Villaeneuve-Aguilar et al., 2019). Likewise, smax1 (or smax1 smxl2) and smxl6,7,8 mutants suppress different max2 phenotypes that are associated with KAR/KL and SL insensitivity, respectively (Stanga et al., 2013, 2016; Soundappan et al., 2015; Wang et al., 2015; Swarbreck

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**Figure 1.** D14 inhibits hypocotyl growth after GR24 treatment via SMAX1 and SMXL2.

(A) Images of representative 5-day-old seedlings of Col-0 (wild type), kai2, d14-1, kai2 d14-1, smax1 smxl2, kai2 smax1 smxl2, d14-1 smax1 smxl2, smxl6,7,8, kai2 smxl6,7,8, and d14-1 smxl6,7,8 grown under continuous red light for 4 days on 0.5x MS agar medium containing 1 μM KAR2, 1 μM rac-GR24, or acetone. Bar, 5 mm.

(B) Hypocotyl lengths of the seedlings shown in (A).

(C) Hypocotyl lengths of 5-day-old seedlings of Col-0, kai2, d14-1, smax1, smxl2, kai2 smax1, d14-1 smax1, kai2 smxl2, and d14-1 smxl2 grown under continuous red light for 4 days on 0.5x MS medium containing 1 μM KAR2, 1 μM rac-GR24, or acetone. Box-and-whisker plots with the same letter are not significantly different from one another (Tukey’s honest significant difference [HSD], p < 0.05, n ≥ 30).

SUPPRESSOR OF MAX2 1 (SMAX1)-LIKE (SMXL) family that are known as DWARF53 (D3) in rice (Oryza sativa) or SMXL6, SMXL7, and SMXL8 in Arabidopsis thaliana (Gomez-Roldan et al., 2008;
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et al., 2019; Villaéçija-Aguilar et al., 2019). In some cases, however, such as drought resistance or mesocotyl elongation, both pathways may influence a trait (Li et al., 2020b; Zheng et al., 2020). Third, promoter-swapping experiments show that KAI2 and D14 are not interchangeable genes whose unique roles arise from different expression patterns (Waters et al., 2015a; Carbonnel et al., 2020b). Fourth, D14 and KAI2 prefer to interact with different SMXL targets (Yao et al., 2017; Khosla et al., 2020b; Wang et al., 2020b; Zheng et al., 2020). Receptor-SMXL interaction specificity is linked to the central D1M domains of SMXL proteins (Khosla et al., 2020b). Fifth, KAR treatment triggers degradation of SMAX1-type, but not D53-type, SMXL proteins (Jiang et al., 2013; Wang et al., 2015; Khosla et al., 2020b; Zheng et al., 2020). Transient co-expression of SL and KAR/KL signaling components from Nicotiana benthamiana also suggests the specific degradation of SMAX1 by KAI2 and a D53-type SMXL by D14 (Carbonnel et al., 2020a). Finally, evolutionary analysis indicates that D14 was derived from KAI2 and D53-type SMXL proteins were derived from SMAX1-type SMXLs (Bythell-Douglas et al., 2017; Walker et al., 2019). Co-evolution of D14 and D53-type SMXLs may have produced an orthogonal SL signaling pathway.

Recent work has challenged the model of insulated SL and KAR pathways. Genetic studies of lateral root development and root swelling initially implied that KAI2 may target SMXL6, SMXL7, and SMXL8 (Swarbreck et al., 2019). However, lateral root development was later shown to be regulated additively by SL and KAR/KL pathways, putatively with shifting contributions from each at different developmental stages (Villaéçija-Aguilar et al., 2019). The effect of smxl6,7,8 on root swelling, which is KAI2-regulated, has been inconsistent between different labs (Swarbreck et al., 2019; Villaéçija-Aguilar et al., 2019). Thus, there is no strong support for KAI2-SMXL6,7,8 crosstalk. By contrast, there is compelling biochemical evidence that D14 can target SMXL2 (Wang et al., 2020b). SMXL2 co-immunoprecipitates D14 in the presence of GR24SDS or GR244DO, synthetic SL analogs of the natural SLs 5-deoxystrigol (5DS) and 4-deoxyorobanchol (4DO). Furthermore, GR244DO promotes the polyubiquitination and degradation of SMXL2 through D14 in the kai2 background (Wang et al., 2020b). This indicates that one-way crosstalk between the SL and KAR pathways is possible, while also raising the question of whether it occurs naturally.

Co-immunoprecipitation of D14 by SMAX1 was not observed, and it is unknown whether D14 can stimulate SMAX1 degradation (Wang et al., 2020b). However, the potential for D14-SMAX1 crosstalk has been suggested by D14-mediated effects of GR24 on hypocotyl elongation, root-hair density, and root-hair elongation, which are controlled by SMAX1 and SMXL2 (Waters et al., 2012; Toh et al., 2014; Stanga et al., 2016; Villaéçija-Aguilar et al., 2019). We investigated whether D14 can interact with SMAX1 and target it for degradation. Here, we report that KAI2-independent hypocotyl inhibition in the presence of an SL analog is genetically dependent on D14 and MAX2 and is primarily due to the destabilization of SMAX1. Although the ability of D14 to interact with SMAX1 and SMXL2 may be a little-used vestige of its evolution from KAI2, this crosstalk has physiological relevance for osmotic stress responses in seedlings.

RESULTS

Genetic evidence for D14 crosstalk with SMAX1 and SMXL2 in seedlings

KAR1, KAR2, and rac-GR24 (a racemic mixture of GR24DS and GR24ent-DS) inhibit hypocotyl elongation of Arabidopsis seedlings grown under continuous red light (Nelson et al., 2010). GR24DS has a D-ring in the stereochemical configuration of natural SLs and signals through D14. Its enantiomer, GR24ent-DS, has a D-ring configuration that is not found in SLs. GR24ent-DS signals mostly through KAI2 but can also activate D14 in vitro and in vivo (Scaffidi et al., 2014; Waters et al., 2015a; Flematti et al., 2016). Although kai2 seedlings are insensitive to KAR2 and mostly insensitive to GR24ent-DS, responses to rac-GR24 and GR24DS remain (Waters et al., 2012; Scaffidi et al., 2014). We first tested whether KAI2-independent responses to GR24 require MAX2. rac-GR24 and GR24DS had no effect on the kai2 max2 hypocotyl, confirming that responses to these compounds are MAX2-dependent (Supplemental Figure 1).

We next examined genetic interactions among kai2, d14-1, smax1, and smxl mutants to determine which SMXL genes are epistatic to KAI2 and D14 (Figures 1A and 1B; Supplemental Figure 2). As shown previously, d14-1 showed wild-type hypocotyl elongation under control conditions, implying that endogenous SLs do not affect hypocotyl growth. By contrast, kai2 had elongated hypocotyls, and smax1 smxl2 hypocotyls were very short (Waters et al., 2012; Stanga et al., 2016). The kai2 d14-1 double mutant was similar to kai2 but was also insensitive to GR24 treatments, indicating that KAI2-independent responses to GR24 occur through D14 (Scaffidi et al., 2014). The kai2 smax1 smxl2 and d14-1 smax1 smxl2 triple mutants showed dramatically decreased hypocotyl lengths that were not further affected by KAR2 or GR24 treatments, similar to smax1 smxl2. This indicated that SMAX1 and SMXL2 are epistatic to KAI2 (Figures 1A and 1B).

Because hypocotyl elongation of d14-1 is similar to that of the wild type, however, the d14-1 smax1 smxl2 triple mutant did not clarify whether SMAX1 and SMXL2 also act downstream of D14 or function in a separate pathway. We found evidence for the former idea by excluding a role for SMXL6, SMXL7, and SMXL8 in hypocotyl growth. We did not observe an appreciable difference between smxl6,7,8 and wild-type seedlings under the mock condition or in their responses to KAR2 or GR24 (Figures 1A and 1B). Moreover, smxl6,7,8 mutations did not substantially affect the length of kai2 or d14-1 hypocotyls under the mock condition or their responses to KAR2 and GR24 treatments, in clear contrast to smax1 smxl2. Therefore, D14-mediated responses to rac-GR24 and GR24DS in seedling hypocotyls are not due to SMXL6,7,8 degradation. Instead, D14 is likely to target SMAX1 and/or SMXL2 for degradation in the presence of GR24.

SMAX1 is the primary regulator of hypocotyl growth targeted by KAI2 and D14

Given the biochemical evidence for D14 interactions with SMXL2, but not SMAX1, we hypothesized that D14 may target SMXL2 for degradation more effectively than SMAX1 (Wang
et al., 2020b). To assess whether KAI2 and D14 differentially target SMAX1 and SMXL2 during hypocotyl elongation, we compared the growth of d14-1 smax1, d14-1 smxl2, kai2 smax1, and kai2 smxl2 seedlings (Figure 1C). Consistent with the larger role of SMAX1 in hypocotyl elongation, smax1 dramatically suppressed the elongated hypocotyl phenotype of kai2, whereas smxl2 had little effect (Stanga et al., 2016). Responses to rac-GR24 and GR245DS were similarly strong in kai2 smxl2 and kai2, putatively reflecting the ability of D14 to act upon SMAX1 (Figure 1C and Supplemental Figure 3).

Interestingly, the average hypocotyl length of seedlings treated with rac-GR24 was slightly shorter for kai2 smax1 (in which D14 and SMXL2 remain) than for d14-1 smax1 (in which KAI2 and SMXL2 remain), suggesting that D14 may target SMAX1 better than KAI2. Conversely, the hypocotyl length of seedlings treated with rac-GR24 was slightly longer for kai2 smxl2 than for d14-1 smxl2, suggesting that KAI2 may target SMAX1 better than D14 (Figure 1C). A similar pattern of results was observed in treatments with purified GR24 stereoisomers (Supplemental Figure 3).

Figure 2. SL triggers SMAX1 and SMXL2 degradation through D14.
(A–C) Relative fluorescence from the SMAX1-mScarlet-I reporter (A), the SMXL2-mScarlet-I reporter (B), or the SMAX1D2-mScarlet-I reporter (C) and the Venus reference after transient expression of the ratiometric system in wild-type (WT) tobacco and Nbd14 is shown. Leaf discs were treated with acetone, 10 μM KAR1, or 10 μM GR245DS for 12 h before measurement. n = 5–8 leaf discs. Asterisks indicate significant differences from each acetone control or between compared pairs using Student’s t test (*p < 0.05 and **p < 0.01).
(D and E) Relative fluorescence from the SMAX1-mScarlet-I reporter (D) or the SMXL7-mScarlet-I reporter (E) along with D14, d14 seto, d14 S97A, or an empty vector (EV) expressed in Nbd14 at 0, 1, and 2 h after 10 μM GR245DS treatment. n = 12 leaf discs. ns, no significance. *p < 0.05, **p < 0.01, Student’s t test comparisons with the relative fluorescence at 0 h or between compared pairs.
(F and G) SMAX1D2-luciferase (LUC) transgenic seedlings in the Col-0, kai2, d14-1, and max2 backgrounds were treated with 5 μM KAR2 (F), 5 μM GR245DS (G), or acetone for 4 h. Bioluminescence is shown as relative LUC activity at 0, 2, and 4 h after treatment. n = 12–14 seedlings. *p < 0.05, **p < 0.01, Student’s t test comparisons with each genotype/treatment at 0 h.
Figure 3. D14 and SMAX1 proteins can physically interact.

(A) Yeast two-hybrid assays for D14 and d14 seto interactions with SMAX1, SMXL7, and their D1M domains. D14 and d14 seto were fused to GAL4-BD. SMAX1, SMXL7, and their domains were fused to GAL4-AD. Serial 10-fold dilutions of yeast cultures were spotted onto selective growth medium (-L, -Leu; -T, -Trp; -H, -His; -A, -Ade) supplemented with 2 μM GR24^DOS^ or acetone (control).

(B–F) Split-LUC complementation assay for interactions between SMXL7 (B), SMAX1_{ΔRGKT} (C), SMXL2 (D), and D1M domains of SMAX1 (E) and SMXL7 (F) with D14, d14 seto, or d14^{S97A}. N. benthamiana leaves were transiently co-transformed with Agrobacterium tumefaciens strains carrying cLUC, nLUC,
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**SMAX1 is degraded after GR24\textsuperscript{SDS} treatment by D14-SCP\textsuperscript{MAX2} signaling**

We next used a ratiometric reporter system to investigate whether D14 can induce degradation of Arabidopsis SMAX1 and SMXL2 proteins (Figure 2) (Khosla et al., 2020a, 2020b). We transiently expressed pHATIQ1212-SMAX1, -SMXL2, and -SMAX1\textsubscript{D2} (a C-terminal domain of SMAX1 sufficient for degradation; see below) dual-fluorescent reporter constructs in wild-type N. benthamiana leaves and tested the effects of 10 \(\mu\text{M}\) KAR1 and GR24\textsuperscript{SDS} treatments on excised leaf discs. The ratio of mScarlet-I/Venus fluorescence decreased for all constructs in response to both treatments, indicating the degradation of SMAX1, SMXL2, and SMAX1\textsubscript{D2}-mScarlet-I fusion proteins (Figures 2A–2C). The extent of degradation induced by GR24\textsuperscript{SDS} was similar to that induced by KAR1. Although GR24\textsuperscript{SDS} responses are predominantly mediated by D14 in Arabidopsis, we could not assume that GR24\textsuperscript{SDS}-induced degradation of SMAX1 and SMXL2 in N. benthamiana was due to D14 alone. Therefore, we also tested these constructs in an N. benthamiana d14a d14b double mutant (\textit{Nbd14}) background (White et al., 2021). The SMXL7 reporter was unaffected by rac-GR24 in \textit{Nbd14}, indicating that its degradation is specifically mediated by \textit{N. benthamiana} D14 proteins and not by KAI2 (White et al., 2021). In \textit{Nbd14} leaves, we observed 55% and 51% less degradation of SMAX1 and SMXL2 reporters, respectively, after 12 h treatment with GR24\textsuperscript{SDS} compared with KAR1 (Figures 2A–2B). At an earlier 4-h time point, GR24\textsuperscript{SDS} had very little effect on SMAX1 degradation compared with KAR1 in the \textit{Nbd14} mutant, but it was effective in the wild type (Supplemental Figure 4). These results indicated that \textit{N. benthamiana} D14 proteins mediate much, although not all, of the GR24\textsuperscript{SDS}-induced degradation of SMAX1 and SMXL2.

To verify that D14 can cause SMAX1 degradation, we rescued the \textit{Nbd14} mutant by transient expression of Arabidopsis D14. As a negative control, we tested the d14\textsuperscript{597A} mutant, which has no SL hydrolase or signaling activity (Waters et al., 2015a; Seto et al., 2019). We also tested the set5/d14-2 allele of Arabidopsis D14 (referred to here as d14\textsuperscript{set5}) to avoid confusion with the \textit{Osds14-2} allele in rice (Chevalier et al., 2014). Co-expression of D14 restored the degradation of SMAX1 and SMXL7 reporters following GR24\textsuperscript{SDS} treatment in \textit{Nbd14} leaves (Figures 2D and 2E). By contrast, d14\textsuperscript{597A} failed to restore GR24\textsuperscript{SDS}-induced degradation of SMAX1 and SMXL7. Interestingly, d14\textsuperscript{set5} enabled GR24\textsuperscript{SDS}-induced degradation of SMAX1 and SMXL7, similar to D14. Moreover, in the absence of GR24\textsuperscript{SDS} treatment, d14\textsuperscript{set5} co-expression reduced the accumulation of the SMAX1 reporter relative to D14 co-expression (Figures 2D and 2E). Therefore, the d14\textsuperscript{set5} allele does not cause a complete loss of function and may be more effective at triggering SMAX1 degradation.

We next investigated whether SMAX1 degradation in Arabidopsis also involves D14. We have not yet been successful in detecting full-length SMAX1 in Arabidopsis (Khosla et al., 2020b). However, the C-terminal D2 domain of SMAX1 (SMAX1\textsubscript{D2}) is more stable than SMAX1 and is necessary and sufficient for MAX2-mediated degradation if full-length SMAX1 and/or SMXL2 proteins are also present. SMAX1\textsubscript{D2} lacks the central D1M domains that mediate interactions between SMXL proteins and their receptor partners, KAI2 or D14, and it is therefore likely to be targeted for degradation indirectly through association with SMAX1 or SMXL2 (Khosla et al., 2020b). D14-mediated, GR24\textsuperscript{SDS}-induced degradation of the SMAX1\textsubscript{D2} ratiometric reporter in \textit{N. benthamiana} was similar to that of the full-length SMAX1 and SMXL2 reporters (Figure 2C). Therefore, we crossed kai2 and d14-1 mutations into a stable transgenic SMAX1\textsubscript{D2}-luciferase (LUC) reporter line in Arabidopsis to analyze KAR2- and GR24\textsuperscript{SDS}-induced degradation responses (Khosla et al., 2020b). KAR2 caused a significant decline in SMAX1\textsubscript{D2}-LUC bioluminescence within 4 h in wild-type and d14-1 seedlings but had no effect on kai2 or max2 seedlings (Figure 2F). By contrast, GR24\textsuperscript{SDS} caused a decline in the abundance of SMAX1\textsubscript{D2}-LUC reporters in wild-type and kai2 seedlings but not in d14-1 or max2 seedlings (Figure 2G). This result demonstrated that GR24\textsuperscript{SDS}-induced degradation of SMAX1\textsubscript{D2} (and, by proxy, SMAX1 and/or SMXL2) in Arabidopsis is due to D14 and MAX2 activity.

**GR24\textsuperscript{SDS} promotes interactions of D14 with SMAX1 and SMXL2**

To determine whether D14 targets SMAX1 and SMXL2 directly, we investigated interactions among these proteins. In yeast two-hybrid (Y2H) assays, GR24\textsuperscript{SDS} stimulated protein-protein interactions between D14 and SMAX1, SMXL2, and SMXL7. Based upon the relative growth rates of yeast under low-stringency histidine dropout selection, D14-SMAX1 interactions were weaker than D14-SMXL2 and D14-SMXL7 interactions and not very different from a GAL4 activation domain (AD) negative control. In the presence of GR24\textsuperscript{SDS}, D14 had stronger interactions with the D1M domains of SMAX1 and SMXL7 (SMAX1\textsubscript{D1M} and SMXL7\textsubscript{D1M}) than the full-length proteins, as indicated by yeast growth under higher-stringency histidine and adenine dropout selection. Again, D14 showed a stronger interaction with SMXL7\textsubscript{D1M} than with SMAX1\textsubscript{D1M} (Figure 3A). As a negative or the induced fusions, as well as a strain carrying an mCherry transgene as a transformation control. Luminescence was measured before and 1 h after treatment with 10 \(\mu\text{M}\) GR24\textsuperscript{SDS} and was normalized against mCherry fluorescence. Box-and-whisker plots with the same letter are not significantly different from one another (Student’s t test, \(p < 0.05\), \(n = 7–15\) leaf discs).

**G** FRET-ABP assay for interactions of SMAX1 with D14. N. benthamiana leaves were transiently co-transformed with \textit{Agrobacterium tumefaciens} strains carrying SMAX1-GFP-mCherry or the indicated fusions. The FRET efficiency is shown as the percentage increase in donor fluorescence compared with that before receptor bleaching. + (dark green box) and – (white box) indicate SMAX1-GFP-mCherry as a positive control and the SMAX1-mCherry/Myc-GFP pair as a negative control, respectively. Acetone-treated leaf discs were used as mock controls. Box-and-whisker plots with the same letter are not significantly different from one another (Student’s t test, \(p < 0.05\), \(n = 6–21\) leaf discs).

**H** FRET-ABP assay for interactions of SMXL7 with D14. N. benthamiana leaves were transiently co-transformed with \textit{Agrobacterium tumefaciens} strains carrying SMXL7-GFP-mCherry or the indicated fusions. + (dark green box) and – (white box) indicate SMXL7-GFP-mCherry as a positive control and the SMXL7-mCherry/Myc-GFP pair as a negative control, respectively. Acetone-treated leaf discs were used as mock controls. Box-and-whisker plots with the same letter are not significantly different from one another (Student’s t test, \(p < 0.05\), \(n = 6–18\) leaf discs).
control, we tested d14S97A and observed no interactions (Supplemental Figure 5).

We also investigated SMAX1 and SMXL7 interactions with d14seto, which has an amino acid substitution at the solvent-exposed surface of a D14 cap helix that may influence protein-protein interactions (Chevalier et al., 2014). We observed that d14seto had highly reduced or abolished Y2H interactions with SMAX1, SMXL2, SMXL7, SMXL7D1M, and the GAL4 AD itself in the presence of GR245DS compared with D14. Unexpectedly, d14seto maintained the interaction with SMAX1D1M and, furthermore, interacted with SMAX1D1M in the absence of GR245DS (Figure 3A).

To validate the Y2H results in a plant system, we examined D14 interactions with SMXL proteins using split-LUC assays in N. benthamiana leaves. N- and C-terminal portions of firefly LUC were fused, respectively, to the C-termini of SMXL proteins and the N-termini of D14, d14seto, or d14S97A. To normalize transformation efficiencies across samples, the fluorescent protein mCherry was co-expressed with the split-LUC constructs. These assays were performed in Nbd14 leaves to avoid possible interference from native NbD14 proteins. The ratio of LUC to mCherry signal produced by cLUC-D14 and SMXL7-nLUC was significantly higher than that produced with unfused cLUC or nLUC negative controls. GR245DS further increased the LUC/mCherry ratio for D14-SMXL7, consistent with enhanced protein-protein interaction. Although d14S97A produced a similar interaction with SMXL7 as D14 before treatment, GR245DS had no effect (Figure 3B). In contrast to the Y2H experiments, d14seto appeared to interact with SMXL7 similarly to D14, albeit with a putatively reduced response to GR245DS. We next tested D14 interactions with SMAX1 and SMXL2. We were unable to detect a LUC/mCherry signal for D14-SMAX1 above that of the negative controls, even in the presence of GR245DS (Supplemental Figure 6). This may reflect the instability of SMAX1 (Khosla et al., 2020b). Deletion of a conserved P-loop motif (RGKT) causes resistance to SCRSMXL-mediated degradation in D53-type SMXL proteins as well as SMAX1 and SMXL2 (Jiang et al., 2013; Zhou et al., 2013; Soundappan et al., 2015; Wang et al., 2015, 2020b; Liang et al., 2016; Khosla et al., 2020b). Therefore, we tested interactions between SMAX1RGKT and D14. This enabled the detection of a GR245DS-responsive interaction with D14, although with a much lower signal than D14-SMXL7 or D14-SMXL2. SMAX1RGKT and SMXL2 interactions with D14, d14seto, and d14S97A were qualitatively similar to those observed for SMXL7, with a positive GR245DS response maintained for d14seto but not for d14S97A (Figures 3C and 3D). SMAX1D1M and SMXL7D1M showed a pattern of interactions with D14 and d14 mutant proteins that was similar to that of full-length SMXL proteins but produced stronger luminescence signals (Figures 3E and 3F, Suplemental Figure 7). In contrast to the Y2H experiments, we did not observe reduced interactions between SMXL7D1M and d14seto compared with D14 in the split-LUC assays.

The differing results in Y2H and split-LUC assays led us to further examine D14 and d14seto interactions with SMAX1 and SMXL7 by measuring Förster resonance energy transfer after acceptor photobleaching (FRET-APB) (Day et al., 2001). This technique determines FRET efficiency, which is a measure of protein-protein interactions, by comparing the fluorescence of the donor (e.g., GFP) before and after photobleaching of the acceptor (e.g., mCherry). We performed FRET-APB assays with D14-GFP, d14seto-GFP, and SMAX1-mCherry fusion proteins co-expressed in N. benthamiana leaves. Photobleaching of SMAX1-mCherry caused a negligible change in fluorescence of a myc-GFP negative control, indicating an absence of FRET between these two proteins (Figure 3G). By contrast, FRET was detected between D14-GFP and SMAX1-mCherry. After 5 min of treatment with a solvent control, SMAX1-mCherry photobleaching caused a small increase in D14-GFP fluorescence. Treatment with GR245DS for 5 or 30 min increased the FRET efficiency approximately 2- to 3-fold above the solvent control. Similar results were obtained for d14seto-GFP and SMAX1-mCherry. The average FRET efficiency between d14seto-GFP and SMAX1-mCherry was higher than that between D14-GFP and SMAX1-mCherry after 30 min of GR245DS treatment (10.4% versus 6.9%), although this difference was not statistically significant ($p = 0.24$, Student’s t test). We also examined D14-SMXL7 interactions with FRET-APB. The FRET efficiency between D14 and SMXL7 peaked within 5 min of GR245DS treatment. Similar FRET efficiencies in the presence and absence of GR245DS were observed between d14seto and SMXL7 (Figure 3H).

Together, these experiments indicate that D14 and SMAX1 can associate in the presence of GR245DS. Y2H and split-LUC experiments suggest that D14 can interact better with SMXL2 than with SMAX1, although this may be due, at least in part, to the instability of SMAX1 (Figure 3 and Supplemental Figure 7). The effect of d14seto is less clear. Although Y2H experiments suggested that d14seto was less able to interact with SMAX1, SMXL2, and SMXL7, this was not supported by split-LUC and FRET-APB assays in plants. The differences could be a consequence of overexpression or of the effects of other proteins in the plant cell environment (e.g., MAX2) on D14 signaling, interactions, and stability. Regardless, d14seto was not as deficient as d14S97A in its interactions with SMXL proteins or the GR245DS response, suggesting that it is hypomorphic rather than amorphic.

A hypomorphic d14 protein is more active when SMAX1 and SMXL2 are absent

Although D14 can induce degradation of SMAX1 and SMXL2, it is unclear whether this is only an artifact of treatments with an exogenous SL analog. If D14-mediated degradation of SMAX1 and SMXL2 has physiological significance, we can expect d14 to affect growth processes controlled by SMAX1 and SMXL2 and/or SMAX1 smxl2 to at least partially suppress d14 phenotypes. As noted above, d14-1 seedlings are phenotypically similar to the wild type. We found that d14seto hypocotyls were slightly shorter than those of the wild type, suggesting that SMAX1/SMXL2 may be partially reduced (Supplemental Figure 8). However, d14seto and kai2 d14seto showed little response to GR245DS, implying that any such targeting by d14seto may reflect promiscuous activity rather than an SL response, as suggested by the Y2H results (Figure 3A and Supplemental Figure 8).

We next examined the effects of kai2, SMAX1, and SMXL2 on the excess shoot-branching phenotype of d14. A recent study based on the overexpression of SMAX1 proposed that SMAX1 suppresses axillary shoot branching (Zheng et al., 2021). Contrary to this result, we did not observe any effect of kai2.
which overaccumulates SMAX1 and SMXL2, or smax1 smxl2 on
the excess branching phenotype of d14-1 (Figures 4A and 4B). We also investigated genetic interactions between d14<sup>seto</sup> and KAR signaling mutants. The excess branching phenotype of d14<sup>seto</sup> was weaker than that of d14-1, consistent with d14<sup>seto</sup> causing a partial loss of function. Interestingly, branching number was increased to d14-1 and max2 levels in the kao2 d14<sup>seto</sup> mutant and reduced in d14<sup>seto</sup> smax1 smxl2. Because max2 was epistatic in the d14<sup>seto</sup> smax1 smxl2 max2 mutant, SMAX1 and SMXL2 are unlikely to regulate shoot branching downstream of MAX2. Instead, these data suggest that SMAX1 and SMXL2 negatively affect the ability of d14<sup>seto</sup> to target SMXL6, SMXL7, and SMXL8 for degradation.

We found further support for this idea from analysis of BRANCHED1 (BRC1) expression in non-elongated axillary buds. BRC1 is a transcription factor that represses axillary bud outgrowth and whose expression is negatively regulated by SMXL6, SMXL7, and SMXL8 (Aguilar-Martínez et al., 2007; Soundappan et al., 2015; Wang et al., 2020a). Consistent with the shoot-branching data, smax1 smxl2 did not increase BRC1 expression in the d14-1 background. BRC1 expression was higher in d14<sup>seto</sup> buds than in d14-1 buds, and the addition of smax1 smxl2 mutations further increased BRC1 expression in a MAX2-dependent manner (Figure 4C).

**SMAX1 and SMXL2 may enhance D14 turnover after SL perception**

One way that SMAX1 and SMXL2 might affect the activity of d14<sup>seto</sup> is by reducing its abundance. D14 and KAI2 are both degraded within hours after activation (Chevalier et al., 2014; Waters et al., 2015b; Hu et al., 2017). KAI2 degradation after KAR treatment is MAX2-independent and probably occurs through association with SMAX1 and SMXL2, which are unstable (Waters et al., 2015b; Khosla et al., 2020b). D14 degradation after GR24 treatment is MAX2-dependent in Arabidopsis (Chevalier et al., 2014). If d14<sup>seto</sup> is more prone to interactions with SMAX1 (Figures 3A and 3C), however, it may undergo increased turnover compared with wild-type D14. This led us to test the degradation of D14-GFP and d14<sup>seto</sup>-GFP fusions expressed in wild-type seedlings after treatment with rac-GR24. We observed a faster rate of decline for d14<sup>seto</sup>-GFP than for D14-GFP in both hypocotyl and root tissues of seedlings after rac-GR24 treatment (Figure 5A).

To assess whether SMAX1 and SMXL2 influence GR24<sup>DS</sup>-induced degradation of D14, we next introduced a UBQ:D14-LUC transgene into wild-type and smax1 smxl2 backgrounds. The decline in bioluminescence from D14-LUC following GR24<sup>DS</sup> treatment was slowed in smax1 smxl2 at all time points.

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**Figure 4. d14<sup>seto</sup> is hypomorphic and more active in an smax1 smxl2 background.**

(A) Adult shoot morphology of Col-0, d14-1, kao2 d14-1, d14-1 smax1, d14-1 smax1 smo2, d14<sup>seto</sup>, kao2 d14<sup>seto</sup>, d14<sup>seto</sup> smax1, d14<sup>seto</sup> smax1 smo2, d14<sup>seto</sup> smax1 smo2 max2, and max2 plants. Bar, 5 cm.

(B) The number of primary rosette branches of plant materials in (A). Box- and whisker plots with the same letter are not significantly different from one another (Tukey’s HSD, p < 0.05, n = 21–34).

(C) qRT-PCR analysis of BRC1/TCP19 gene expression in non-elongated axillary buds of Col-0, d14-1, d14-1 smax1 smo2, d14<sup>seto</sup>, kao2 d14<sup>seto</sup>, d14<sup>seto</sup> smo2, d14<sup>seto</sup> smo2 max2, and max2 plants collected 10 days after anthesis. Expression of BRC1 is relative to the CACS internal reference gene. Scatter dot plots with the same letter are not significantly different from one another (bar indicates mean; n = 4 pooled tissue samples, three plants per pool; Student’s t test, p < 0.05).
Crosstalk between D14 and SMAX1

**Figure 5. D14 degradation after GR24_{5DS} treatment is enhanced by SMAX1 and SMXL2.**

(A) The relative GFP signal from D14-GFP or d14seto-GFP transgenic plants was measured every 10 min in the presence of 5 μM rac-GR24. The curve was generated from the mean value per genotype/treatment at each time point. Bar indicates SE of the mean (n = 6 seedlings).

(B) UBP:D14-LUC transgenic seedlings in the Col-0 and smax1 smxl2 backgrounds were treated with 5 μM GR24_{5DS} or acetone for 12 h. Bioluminescence is shown as relative LUC activity at 0, 2, 4, 8, and 12 h after treatment. n = 10–12 seedlings. Asterisks indicate significant differences relative to each group at 0 h or between compared pairs using Student’s t test (*p < 0.05 and **p < 0.01; ns, no significance).

(C) Time-course assay of D14, d14seto, and d14_{S97A} stability in *N. benthamiana* under 10 μM GR24_{5DS} treatment. Relative fluorescence from the D14-mScarlet-I reporter, the d14seto-mScarlet-I reporter, or the d14_{S97A}-mScarlet-I reporter and the Venus reference after transient co-expression of the ratiometric system and SMAX1 effector in tobacco is shown. Leaf discs were treated for 12 h to monitor D14, d14seto, and d14_{S97A} stability. n = 14 leaf discs. Asterisks indicate significant differences relative to each group at 0 h or between compared pairs using Student’s t test (*p < 0.05 and **p < 0.01; ns, no significance).

D14 has no effect on SMAX1 and SMXL2 degradation in response to endogenous SLs during seedling photomorphogenesis or shoot branching. We reasoned that D14-mediated degradation of SMAX1 and SMXL2 might be physiologically relevant for some traits regulated by both pathways or under conditions in which endogenous SL levels are sufficiently high. SL biosynthesis genes are induced by dehydration or mild drought in Arabidopsis and rice, leading to increased SL, at least in rice roots (Van Ha et al., 2014; Haider et al., 2018).

Therefore, as an alternative means of imposing drought/water deficit, we examined the response of KAR and SL signaling pathway mutants to osmotic stress. Wild-type seedlings grown in the presence of 300 mM mannitol showed a 40% reduction in fresh weight compared with seedlings grown on standard medium (Figures 6A and 6B). Growth inhibition by mannitol was enhanced in d14 and kai2 seedlings, and mannitol also caused a reduction in chlorophyll content (Figures 6A and 6C). We found that smxl6,7,8 seedlings were even more strongly affected by mannitol than kai2 and d14. By contrast, smxl1 smxl2 seedlings were resistant to mannitol, showing only a 10% reduction in fresh weight and an increase in chlorophyll content under mannitol treatment. Intriguingly, SMAX1 and SMXL2 contributed differently to osmotic stress tolerance. Under mannitol treatment, we observed less reduction in biomass in smax1 seedlings and higher chlorophyll content in smxl2 seedlings compared with the wild type (Supplemental Figure 9).

**D14-SCF_{MAX2} mediates SMAX1 degradation induced by osmotic stress**

Although D14 and KAI2 often affect different developmental traits, this is not always the case. For example, both the SL and KAR/KL pathways promote drought tolerance in Arabidopsis (Li et al., 2017, 2020a, 2020b; Haider et al., 2018). Our data suggest that compared with the wild type, suggesting that D14-LUC was partially stabilized by the absence of SMAX1 and SMXL2 (Figure 5B). We then transiently expressed D14, d14_{seto}, and d14_{S97A} ratiometric reporters with or without Arabidopsis SMAX1 in Nbd14 leaves (Figure 5C). The d14_{S97A} reporter was the most stable of the three variants; it showed the highest relative abundance and was unaffected by GR24_{5DS} treatment. D14 and d14_{seto} reporters both declined in the 12 h after GR24_{5DS} treatment. As in Arabidopsis, d14_{seto} showed a faster rate of decline in tobacco. Co-expression of SMAX1 caused a small but significant increase in GR24_{5DS}-induced turnover of D14 at two time points and of d14_{seto} at all time points. This suggested that the interaction of D14 with SMAX1 and SMXL2 may reduce its abundance in the presence of GR24; increased availability of a partially active d14_{seto} protein may explain why the d14_{seto} smax1 smxl2 mutant showed partially recovered shoot branching.
Figure 6. D14 targets SMAX1 and SMXL2 under osmotic stress.
(A) Twenty-one-day-old seedlings of Col-0, smax1 smxl2, smxl 6,7,8, d14-1, d14-1 smax1 smxl2, d14-1 smxl6,7,8, kai2, kai2 smax1 smxl2, and kai2 smxl6,7,8 grown under mock or 300 mM mannitol conditions for 14 days. Bar, 2 cm.
(B) Relative fresh weights of plant materials used in (A) after application of 300 mM mannitol. The weights of aerial parts from plants grown on 0.5× MS agar medium with 300 mM mannitol are scaled to those from plants grown on 0.5× MS agar medium. Scatter dot plots with the same letter are not significantly different from one another (bar indicates mean; n = 4; Student’s t test, p < 0.05).
(C) Chlorophyll (Chl) contents in the aerial parts of Arabidopsis seedlings used in (A). Others are as in (B).

(legend continued on next page)
Crosstalk between D14 and SMAX1

To assess the effect of the smax1 smxl2 or smxl6,7,8 mutant on osmotic-stress-induced gene expression, we performed quantitative RT-PCR of RD29A, Cor15A, and PKS5 (Fuji et al., 2011; Liu et al., 2014). Induction of RD29A, Cor15A, and PKS5 transcripts in response to mannitol treatment was impaired in smax1 smxl2 seedlings. By comparison, RD29A showed normal upregulation in response to mannitol treatment in smxl6,7,8 seedlings. Cor15A and PKS5 were not as highly induced by mannitol in smxl6,7,8 seedlings than in the wild type but were more highly induced than in smax1 smxl2 (Figure 6E).

Because smax1 smxl2 had phenotypes opposite to those of d14 and kai2 and was epistatic to both, we hypothesized that D14 might contribute to SMAX1 and SMXL2 degradation during mannitol treatment. To test this possibility, we compared degradation of the SMAX1-D2-LUC reporter after mannitol treatment in Col-0, kai2, d14-1, max2, and the SL biosynthetic mutant max3. We observed degradation of SMAX1-D2-LUC within 8 h of mannitol treatment in wild-type and kai2 seedlings but not in d14-1, max2, or SL-deficient max3 seedlings (Figure 6D). SMXL7-LUC was also destabilized in a D14-dependent manner under mannitol treatment, supporting the idea that the level of endogenous SL and/or D14-SCF^MAX2^ signaling is induced by osmotic stress (Supplemental Figure 10). These results suggest that SL-induced degradation of SMAX1 and SMXL2 via D14-SCF^MAX2^ is not just an artificial consequence of GR24 application but can also occur under specific environmental conditions.

DISCUSSION

Although there are strong similarities between the KAR/KL and SL signaling pathways, genetic and biochemical studies have suggested that they are well insulated by specific receptor-target interactions, enabling distinct developmental responses to KAR/KL and SL (SOUNDAPPAN, 2015; VILLAECIJA-AGUILAR et al., 2019). Contradicting this model, here we have shown that D14 can target SMAX1 for degradation after SL analog treatments. Genetic tests indicated that SMAX1 and, to a lesser degree, SMXL2 regulate hypocotyl elongation, but SMXL6, SMXL7, and SMXL8 do not (Figure 1). This result implied that the D14-mediated effect of GR24 on hypocotyl elongation is due to D14-SMAX1 crosstalk. This idea was supported by the observation that an SMAX1 ratiometric reporter was degraded in N. benthamiana after GR24^{DS} treatment in a partially D14-dependent manner (Figure 2A). GR24^{DS} induction of the SMAX1-D2 reporter in Arabidopsis thaliana was also blocked in the d14 background (Figure 2G). Physical interactions between D14 and SMAX1, however, are at best at worst (Figure 3). SMXL proteins, which are distantly related to HSP101 heat-shock proteins that form hexamers, may form multimeric complexes (Khosla et al., 2020b). If heterogeneous complexes form (e.g., composed of SMAX1 and non-SMAX1 subunits), it is possible that SMAX1 could be indirectly targeted for proteolysis by a non-cognate receptor (i.e., D14) that interacts with SMXL2 or SMXL7. However, SMAX1 degradation by D14 does not require the presence of SMXL2, as demonstrated by the GR24 response of kai2 smxl2 seedlings (Figure 2), nor does GR24-induced degradation of SMAX1 and SMXL2 by D14 require SMXL6, SMXL7, or SMXL8, as shown by kai2 smxl6,7,8 seedlings (Figure 1C).

Therefore, our data suggest that a direct interaction between D14-SCF^MAX2^ and SMAX1 can occur when an SL analog is applied. Similarly, D14 can crosstalk with SMXL2 in the presence of SL analogs (WANG et al., 2020b). By contrast, there is no indication that KAR application can cause KA12-SCF^MAX2^ to target D53 or SMXL7 for degradation, and the current genetic evidence for such crosstalk is controversial (Jiang et al., 2013; Wang et al., 2015; SWARBRECK et al., 2019; VILLAECIJA-AGUILAR et al., 2019; Khosla et al., 2020b). We propose an update of the fully insulated KAR/KL and SL signaling models to include one-way promiscuity, in which D14 crosstalk with SMAX1 and SMXL2 is a putative remnant of its evolution from a KAI2 paralog (see below: Figure 7).

SMAX1 can be targeted by D14 in Arabidopsis, but less well than SMXL2

It is likely that D14 has lower affinity for SMAX1 than for SMXL2. Although both SMAX1 and SMXL2 are able to co-immunoprecipitate KA12 from Arabidopsis protoplasts in the presence of an agonist, only SMXL2 is effective at co-immunoprecipitation of D14 (WANG et al., 2020b). SMAX1 also did not interact with D14 in vitro in a pull-down assay (YAO et al., 2017). Likewise, we observed weaker Y2H interactions between D14 and SMAX1 than between D14 and SMXL2 (Figure 3A). In addition, we saw negligible luminance in split-LUC assays for D14-SMAX1 interactions compared with D14-SMXL2 or D14-SMXL7. This may be due to MAX2-dependent and/or-independent degradation of SMAX1 that causes high turnover (Khosla et al., 2020b). The luminance signal was increased in split-LUC assays between D14 and a degradation-resistant SMAX1_{HILIGHT} mutant protein, although it was still weaker than that produced by D14-SMXL2 interactions (Figure 3 and Supplemental Figure 6). Finally, we note that although we observed a strong effect of 500 nM GR24^{DS} on hypocotyl elongation of both smax1 and smxl2, , 2020b observed different D14-mediated responses to 100 nM GR24^{DS} treatments in these mutants (Figure 1 and Supplemental Figure 3). The 100 nM GR24^{DS} treatment had only a small effect on hypocotyl elongation of smxl2 but had a large effect on smax1 seedlings, implying that SMAX1 may be less effectively degraded than SMXL2. Lower concentrations of SL may be required to induce D14 crosstalk with SMXL2 than with SMAX1. For developmental processes such as root-hair elongation, in which SMXL2 has a more prominent role than SMAX1, or root skewing, to which SMXL2 and SMAX1 contribute non-redundantly, endogenous SLs may be more likely to have an
parasitic plants in the Orobanchaceae to sense host-derived SLs and germinate (Nelson, 2021).

One way that selective protein-protein interactions between KAII-SMAX1 and D14-SMXL7 could have evolved is via mutations in an SL-responsive KAII paralog (a proto-D14) that disrupt SMAX1 interactions, combined with compensatory mutations in an SMAX1 paralog (a proto-SMXL7) that establish an orthogonal interaction with the proto-D14. However, this evolutionary path involves an intermediate phase during which the proto-D14 is a pseudogene and/or the proto-SMXL7 is misregulated, with potentially detrimental effects. Bacterial toxin-antitoxin systems have revealed an alternative way in which duplicated protein pairs may evolve selective interactions: via a promiscuous intermediate state (Aakre et al., 2015). According to a promiscuity-based model, proto-D14 might first acquire a mutation that broadens its potential interaction specificity. This would enable proto-SMXL7 to acquire a mutation that blocks interactions with KAII but maintains interactions with proto-D14, without negatively affecting fitness. Subsequently, proto-D14 may acquire another mutation that narrows its interaction specificity to proto-SMXL7 alone. Throughout this process, SMXL7 regulation would continue. Substantial work will be needed to evaluate this hypothesis. However, we propose that the ability of D14 to engage in a non-preferred interaction with SMAX1 could be a remnant of such an evolutionary process.

Effects of the d14seto allele

The d14seto allele, which causes a Pro169Leu substitution, appears to reduce the selectivity of D14 against SMAX1 interactions (Figures 3A and 3C). Pro169 is a highly conserved (>90%) surface residue found within a small motif that distinguishes D14 and KAII proteins (ADV—P versus GDMDS, respectively) (Chevalier et al., 2014; Bythell-Douglas et al., 2017). As such, it has been hypothesized to be a specificity-determining position (Chevalier et al., 2014). Alternatively, this motif may influence SL perception. The motif containing Pro169 comprises most of a short loop that joins the α2 and α3 helices (also known as αE and αF) of D14. The composition of this loop affects the rigidity of the ligand-binding pocket, which in turn affects ligand affinities (Bürger et al., 2019).

Our results suggested that d14seto causes a partial loss of function in SL signaling, as it had weaker branching and leaf morphology phenotypes than the null T-DNA insertion allele d14-1 (Figure 4B and Supplemental Figure 1). This result implied that d14seto was less effective at triggering SL-induced degradation of SMXL6, SMXL7, and SMXL8. However, in transient expression experiments in N. benthamiana, d14seto showed an ability similar to that of wild-type D14 to interact with SMXL7 and cause its degradation (Figures 2E, 3B, 3F, and 3H). Therefore, we hypothesized that the d14seto protein may have reduced function because of higher instability. Supporting this notion, we found that d14seto was more rapidly degraded following GR24 treatment than wild-type D14 in Arabidopsis and N. benthamiana (Figures 5A and 5C).

KAII degradation after KAR treatment is MAX2-independent and is probably driven by association with unstable SMAX1 and/or SMXL2 proteins (Waters et al., 2015b; Khosla et al., 2020b).
found that D14 instability was reduced in the smax1 smxl2 background (Figure 5B), suggesting that it may also be degraded by association with SMAX1 and/or SMXL2. This led us to hypothesize that enhanced d14\textsuperscript{seto} turnover after SL perception might be caused by stronger association with SMAX1 and/or SMXL2 compared with wild-type D14. Indeed, co-expression of SMAX1 slightly enhanced d14\textsuperscript{seto} degradation in N. benthamiana (Figure 5C). This hypothesis also predicts that the phenotypes of d14\textsuperscript{seto} will be affected by SMAX1/SMXL2 abundance. Consistent with this prediction, the branching phenotype of d14\textsuperscript{seto} was increased by the addition of kai2. Overaccumulation of SMAX1 and SMXL2 in kai2 might further reduce d14\textsuperscript{seto} abundance (Figure 4B). Conversely, the excess branching of d14\textsuperscript{seto} was partially suppressed by smax1 smxl2, perhaps indicating that the d14\textsuperscript{seto} protein had been stabilized. Similarly, smax1 smxl2 partially suppressed the reduced BRC1 expression in d14\textsuperscript{seto} (Figures 4B and 4C). By comparison, smax1 smxl2 had no effect on branching or BRC1 expression in the null d14-1 background (Figures 4B and 4C).

The physiological relevance of D14-SMAX1 crosstalk

Although D14 can target SMAX1 and SMXL2 for degradation after SL treatment, SL-deficient and SL-insensitive mutants do not show phenotypes associated with SMAX1 and SMXL2 overaccumulation, suggesting that this crosstalk does not normally occur (Nelson et al., 2011; Waters et al., 2012; Soundappan et al., 2015). Alternatively, SL levels that are sufficiently high to stimulate D14 crosstalk may occur only in limited developmental contexts. SL biosynthesis is induced by various stresses such as drought and phosphate starvation (Löperea-Ráez et al., 2008; Van Ha et al., 2014; Haider et al., 2018). This led us to explore whether D14-SMAX1 crosstalk occurs during water stress. Interestingly, although smxl6,7,8 plants have enhanced resistance to water deficit, opposite to d14 and SL-deficient mutants, we found that smxl6,7,8 seedlings are more susceptible to osmotic stress (Van Ha et al., 2014; Li et al., 2020a, 2020b) (Figures 6A–6C). This was particularly surprising because d14 was also more susceptible to osmotic stress than the wild type. By contrast, smax1 smxl2 had enhanced resistance to osmotic stress and was epistatic to d14 and kai2 for this trait (Figures 6A–6C). Defective induction of RD29A and Cor15A expression in smax1 smxl2 may confer osmotic stress tolerance by strengthening photosynthesis and seedling growth (Msanne et al., 2011; Liu et al., 2014). Alternatively, given that smax1 smxl2 seedlings showed better growth under mannitol treatment than the wild type, the reduced upregulation of RD29A, Cor15A, and PKS5 by mannitol may indicate that smax1 smxl2 is less susceptible to osmotic stress. Although we cannot yet explain smx6,7,8 phenotypes, this result suggested that D14 might target SMAX1 and SMXL2 under osmotic stress. Indeed, we observed enhanced degradation of an SMAX1\textsubscript{PS} reporter following osmotic stress—without GR24 treatments—that was dependent on D14 and the SL biosynthesis gene MAX3. It is also notable that KAR-responsive genes are upregulated under osmotic stress (Shah et al., 2020). This implies a reduction in SMAX1 and SMXL2 levels, which could potentially be due to SL signaling activity. In conclusion, we propose that under some environmental conditions or developmental contexts, D14 crosstalk initiated by SLs may broaden the ability of plants to fine-tune SMAX1 and SMXL2 regulation.

METHODS

**Plant materials**

*Arabidopsis thaliana* mutants d14-1, d14\textsuperscript{seto}, htl-3 (a kai2 allele), d14-1 htl-3, max2-1, smax1-2, smxl2-1, smax1-2 smxl2-1, smxl6-4 smxl7-3 smxl8-1, and max3-11 have been described previously (Waters et al., 2012; Stanga et al., 2013, 2016; Chevalier et al., 2014; Toh et al., 2014; Soundappan et al., 2015). All lines are in the Col-0 ecotype. Genotyping primers are listed in Supplemental Table 1. Detailed methods are found in the supplemental information.

**SUPPLEMENTAL INFORMATION**

Supplemental information is available at Plant Communications Online.

**FUNDING**

We gratefully acknowledge funding support from US National Science Foundation (NSF) grants IOS-1737153, IOS-1740560, and IOS-1856741 to D.C.N. and Spanish Ministry of Science and Innovation grants BIO2017-84363-R and PID2020-112779RB-I00 and FESF Investing in Your Future to P.C.

**ACKNOWLEDGMENTS**

We thank Dr. Gavin Flematti and Dr. Adrian Scaffidi (University of Western Australia) for supplying KAR1, KAR2, rac-GR24, and purified GR24 enantiomers. No conflict of interest declared.

Received: August 3, 2021

Revised: December 15, 2021

Accepted: January 25, 2022

Published: January 31, 2022

**SUPPORTING CITATIONS**

The following references appear in the Supplemental Information: Gietz and Woods, 2002; Goddard-Borger et al., 2007.

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