SUPPRESSOR OF MORE AXILLARY GROWTH2 1 Controls Seed Germination and Seedling Development in Arabidopsis[1][W][OPEN]

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Abiotic chemical signals discovered in smoke that are known as karrikins (KARs) and the endogenous hormone strigolactone (SL) control plant growth through a shared *MORE AXILLARY GROWTH2* (*MAX2*)-dependent pathway. A SL biosynthetic pathway and candidate KAR/SL receptors have been characterized, but signaling downstream of MAX2 is poorly defined. A screen for genetic suppressors of the enhanced seed dormancy phenotype of *max2* in Arabidopsis (*Arabidopsis thaliana*) led to identification of a *suppressor of max2 1* (*smax1*) mutant. *smax1* restores the seed germination and seedling photomorphogenesis phenotypes of *max2* but does not affect the lateral root formation, axillary shoot growth, or senescence phenotypes of *max2*. Expression of three transcriptional markers of KAR/SL signaling, *D14-LIKE2, KAR-UP F-BOX1*, and *INDOLE-3-ACETIC ACID INDUCIBLE1*, is rescued in *smax1 max2* seedlings. *SMAX1* is a member of an eight-gene family in Arabidopsis that has weak similarity to *HEAT SHOCK PROTEIN 101*, which encodes a caseinolytic peptidase B chaperonin required for thermotolerance. *SMAX1* and the *SMAX1-like* (*SMXL*) homologs are differentially expressed in Arabidopsis tissues. *SMAX1* transcripts are most abundant in dry seed, consistent with its function in seed germination control. Several *SMXL* genes are up-regulated in seedlings treated with the synthetic SL GR24. *SMAX1* and *SMXL2* transcripts are reduced in *max2* seedlings, which could indicate negative feedback regulation by KAR/SL signaling. *smax1* seed and seedling growth mimics the wild type treated with KAR/SL, but *smax1* seedlings are still responsive to 2*H*-furo[2,3-*c*]pyran-2-one (KAR_2) or GR24. We conclude that SMAX1 is an important component of KAR/SL signaling during seed germination and seedling growth but is not necessary for all MAX2-dependent responses. We hypothesize that one or more SMXL proteins may also act downstream of MAX2 to control the diverse developmental responses to KARs and SLs.

Strigolactones (SLs) and karrikins (KARs) comprise two classes of butenolide signaling molecules that stimulate seed germination. SLs are exuded from roots into the rhizosphere under nutrient-poor conditions to recruit beneficial symbiotic associations with arbuscular mycorrhizal fungi (Ruyter-Spira et al., 2013). To the seed of obligate root parasites in the family Orobanchaceae (e.g. *Striga* and *Orobanche* spp.), SLs in the rhizosphere signal the presence of a nearby host and induce a potent germination response (Yoneyama et al., 2010). KARs are found in smoke produced by burning plant material and serve an important ecological role as chemical signals that activate post-fire germination in the soil seed bank. KARs enhance germination of many plant species and also have positive effects on seedling vigor and photomorphogenesis (Nelson et al., 2012).

SLs are now recognized as carotenoid-derived plant hormones with a prominent role in the control of axillary shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). Other developmental roles for SLs in the control of secondary growth in the stem, lateral root formation, root hair elongation, primary root length, adventitious root initiation, and senescence have been described in Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), pea (*Pisum sativum*), and petunia (*Petunia hybrida*; Woo et al., 2001; Snowden et al., 2005; Li et al., 2009; Agusti et al., 2011; Kapulnik et al., 2011a, 2011b; Koltai, 2011; Ruyter-Spira et al., 2011; Hamiaux et al., 2012; Rasmussen et al., 2012).

Application of either KAR or the synthetic SL GR24 to Arabidopsis seed and seedlings produces similar effects; both compounds enhance germination, promote responses to light (e.g. reduced hypocotyl elongation), and affect the expression of several transcriptional markers including *D14-LIKE2* (*DLK2*), *KAR-UP F-BOX1* (*KUF1*), and *INDOLE-3-ACETIC ACID INDUCIBLE1* (*IAA1;* Nelson et al., 2009, 2010). However, plants also have distinct responses to these two classes of compounds. GR24 suppresses axillary shoot growth

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in both Arabidopsis and pea SL-deficient mutants, but KAR does not affect shoot branching (Nelson et al., 2011). GR24 inhibits cotyledon expansion in seedlings, but KAR has neutral or positive effects on cotyledon expansion.

Genetic studies have demonstrated that *MORE AXILLARY GROWTH2* (*MAX2*) has a central role in mediating both KAR and SL signaling and controls a broad range of developmental processes (for review, see Janssen and Snowden, 2012). *MAX2* has been identified in genetic screens for Arabidopsis mutants with increased lateral shoot branching (*max2*; Stirnberg et al., 2002), delayed leaf senescence (*oresara9* [*ore9*]; Woo et al., 2001), decreased light responses (*pleiotropic photosignaling*; Shen et al., 2007), and 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (KAR_1)-insensitive germination (*karrikin-insensitive1* [*kai1*]; Nelson et al., 2011). *MAX2* orthologs are required for suppression of axillary shoot growth/tillering by SL in Arabidopsis, pea, and rice (*Oryza sativa*; Gomez-Roldan et al., 2008; Umehara et al., 2008). *max2* seed and seedlings are also entirely insensitive to KAR and SL (Nelson et al., 2011).The rice and pea orthologs of MAX2, DWARF3 (D3), and RAMOSOUS4 (RMS4), respectively, are important for the establishment of arbuscular mycorrhizae (Yoshida et al., 2012; Foo et al., 2013).

As MAX2 is a convergence point for KAR and SL signaling, how are distinct responses produced? Two homologous α/β-hydrolase superfamily proteins, KAI2 and D14, act upstream of MAX2 and are necessary for specific responses to KAR and SL in Arabidopsis. *kai2* and *d14* mutants exhibit unique subsets of *max2* phenotypes at different stages of development; KAI2 regulates seed germination, D14 controls axillary branching, and both proteins can affect seedling growth (Waters et al., 2012b). SL produced from the precursor carlactone signals predominantly through D14, whereas KAI2 is important for carlactone-independent signaling (Scaffidi et al., 2013). Crystal structures have been recently solved for KAI2 and D14 orthologs in Arabidopsis, rice, and petunia (Hamiaux et al., 2012; Bythell-Douglas et al., 2013; Guo et al., 2013; Kagiyama et al., 2013; Zhao et al., 2013). KAI2 and D14 are likely to be receptors that transduce KAR/SL signals through hydrolysis-induced conformational changes. DECREASED APICAL DOMINANCE2, an ortholog of D14 in petunia, has slow hydrolytic activity on GR24 and undergoes hydrolase-dependent shifts in thermal stability in the presence of GR24 (Hamiaux et al., 2012). Zhao et al. (2013) resolved a GR24 degradation intermediate covalently bound to the active site Ser of D14. Most recently, Guo et al. (2013) demonstrated that KAI2 binds KAR_1 and identified resulting conformational changes in KAI2 structure.

Although significant strides have been made in early aspects of KAR/SL signaling, events downstream of MAX2 remain incompletely understood. *MAX2* encodes an F-box protein with C-terminal leucine-rich repeats that is highly conserved among land plants (Waters et al., 2011; Delaux et al., 2012). F-box proteins classically act as adapter components that confer substrate specificity to SKP Cullin F-box E3 ubiquitin-protein ligase complexes. The target proteins recognized by the F-box protein are typically polyubiquitinated and subsequently degraded by the 26S proteasome (Somers and Fujiwara, 2009). Therefore, an inability to degrade one or more substrates of MAX2 may underlie *max2* phenotypes, including the loss of KAR and SL sensitivity. As *max2* mutants exhibit phenotypes in several developmental processes, of which KAR influences only a subset, it is plausible that MAX2 has multiple targets.

Screens for extragenic suppressors of *max2* may reveal early downstream components of the KAR/SL signal transduction pathways or substrates of MAX2. To date, there are three reports of this approach. Stirnberg et al. (2012b) identified *FAR-RED ELONGATED HYPOCOTYL3* (*FHY3*) in a screen for suppressors of the branching phenotype of *max2*. Increased branching in *max2* is correlated with higher basipetal auxin transport in the stem and increased activity of the auxin-responsive reporter *DR5*::*GUS* in the stem vasculature (Bennett et al., 2006; Crawford et al., 2010). Although the *fhy3* mutation reduced axillary branching of *max2*, it did not suppress basipetal auxin transport or *DR5*::*GUS* expression in *max2* stems. FHY3 was proposed to influence axillary branching by regulating auxin homeostasis through AUXIN-RESISTANT1, rather than acting specifically in a MAX2 pathway (Stirnberg et al., 2012b). The cytoplasmic ribosomal protein S10e mutant *rps10b* is a second example of a *max2* suppressor identified through shoot-branching screens. It has a reduced ability to initiate or maintain axillary shoot meristems (Stirnberg et al., 2012a). *rps10b* may suppress a downstream effect of *max2* in bud outgrowth via an auxin-related mechanism. However, *rspb10b* did not restore altered *max2* shoot vascular architecture or antagonize the effect of *max2* on polar auxin transport in the stem (Stirnberg et al., 2012a). An activation-tagging-based screen for suppressors of the delayed leaf senescence phenotype of *ore9* (*max2*) identified *suppressor of ore9 dominant* (*sor1-D*; Hur et al., 2012). This mutation causes overexpression of *AtCHX24*, which encodes a putative cation/H^+ exchanger. *sor1-D* did not restore the axillary branching, inflorescence height, or leaf morphology phenotypes of *ore9*. Notably, *sor1-D* also suppressed the delayed senescence phenotypes of *ore1* and *ore3*, which are thought to act in a different pathway than *ORE9* (Hur et al., 2012). Therefore, FHY3, RPS10B, and *AtCHX24* are not likely to be specific components of the *MAX2* pathway.

We reasoned that *max2* suppressor screens based on axillary branching or senescence phenotypes might be recalcitrant due to functional redundancy among downstream components of the signaling pathway, or an inherent difficulty in distinguishing *MAX2* pathway-specific mutants from many nonspecific suppressors. We therefore undertook a screen for suppressor mutations that restore multiple phenotypes of *max2* seed and seedlings. Here, we report the identification and characterization of *SUPPRESSOR OF MAX2 1* (*SMAX1*).

RESULTS

A Genetic Screen for *suppressor of max2 (smax)* Mutants

To identify genes that may act downstream of MAX2 in KAR/SL signaling pathways, we performed a genetic screen for *smax* mutants. In contrast to previously published *max2* suppressor screens, we chose to search for mutants that alter *max2* phenotypes at early stages of growth. These *max2* phenotypes include increased seed dormancy, modified seedling photomorphogenesis, and altered expression of KAR/SL transcriptional markers (Stirnberg et al., 2002; Shen et al., 2007, 2012; Nelson et al., 2011; Waters et al., 2012b). We reasoned that extragenic mutations that suppress multiple *max2* phenotypes would be most likely to affect signaling components that are specific to the MAX2 pathway in seedlings and act early in KAR/SL response.

The *max2-8* mutant was previously isolated as a frameshift allele of *MAX2* from a γ-ray mutagenized population of Landsberg *erecta* (L*er*) seeds (Nelson et al., 2011). We mutagenized *max2-8* with ethyl methane sulfonate. Approximately 100,000 primary dormant M_2 seeds collected from 2,500 M_1 plants were screened for rapid germination in the presence of KAR. Early germinants with shortened hypocotyls or enlarged cotyledons were preferentially selected. The germination and seedling photomorphogenesis phenotypes of putative suppressors were reexamined in the M_3 generation. One mutant that maintained a low seed dormancy phenotype, *smax1-1 max2-8*, also had reduced hypocotyl elongation and increased cotyledon expansion when grown under continuous red light. Even in the absence of KAR treatment, *smax1-1 max2-8* seedlings mimicked wild-type growth responses to 2*H*-furo[2,3-*c*]pyran-2-one (KAR_2; Supplemental Fig. S1).

Recombination mapping was performed on F2 progeny from an outcross of *smax1-1 max2-8* to the *max2-1* mutant in the Columbia (Col-0) ecotype. Natural variation in seed dormancy between L*er* and Col-0 ecotypes necessitated the confirmation of putative recombinants by seedling morphology as well as seed germination assays. The recessive *smax1-1* mutation was mapped to the bottom of chromosome 5 near marker OXFSSLP454019 (0/290 recombinants), and candidate genes in the region were sequenced. A transition mutation (C→T) that converts Arg-292 into a premature stop codon was identified in the first exon of *At5g57710* in *smax1-1 max2-8* plants and not in *max2-8* plants (Fig. 1A).

We isolated independent transfer DNA (T-DNA) insertion alleles of *At5g57710* in the Col-0 ecotype (Fig. 1A). The *smax1-2* and *smax1-3* insertions lie in the first and fourth exons, respectively. Very little (<2%) *SMAX1* transcript was detected 3′ of the *smax1-3* insertion by quantitative reverse transcription (qRT)-PCR (Fig. 1B). Although *SMAX1* transcript 3′ of the *smax1-2* insertion was only reduced 4-fold, an amplicon surrounding the insertion site could not be generated from *smax1-2* RNA

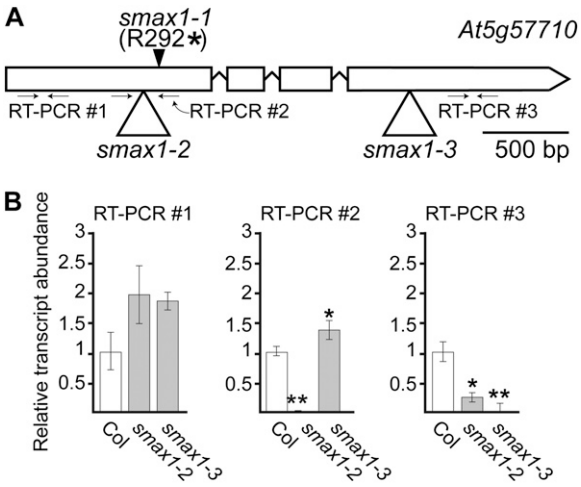

Figure 1. Identification of *smax1* alleles. A, The *smax1-1* mutation (dark triangle) causes a premature stop at codon 292 of *At5g57710*. Four exons shown; large triangles indicate T-DNA alleles (*smax1-2*: SALK_128579; *smax1-3*: SALK_097346). Small arrows indicate primer pair loci for the expression analysis shown in B. B, Abundance of *SMAX1* transcript at three loci detected by real-time reverse transcription-PCR using complementary DNA derived from 7-d-old light-grown seedlings. Expression values are relative to the *CLATHRIN ADAPTOR COMPLEX SUBUNIT* (*CACS*) reference gene and normalized to Col-0 = 1. Mean ± SE (*n* = three independent samples, >50 seedlings per sample). Significance assessed by Student's *t* test (*$P < 0.05$, **$P < 0.01$).

(Fig. 1B). Therefore, *SMAX1* transcripts are disrupted in *smax1-3* and *smax1-2* seedlings. Further experimental work, shown below, was performed with the *smax1* T-DNA insertion alleles, as they are expected to have less mutational load than *smax1-1* and are in a more commonly used genetic background.

smax1 Suppresses Several *max2* Phenotypes during Early Growth Phases

To confirm the identification of *SMAX1*, we generated homozygous *smax1-2 max2-1* and *smax1-3 max2-2* double mutants and tested these lines for suppression of *max2* phenotypes. After 5 d of imbibition at 24°C, wild-type seed germination was approximately 20% on control media and approximately 60% to 70% on 1 μM KAR_2 and 10 μM GR24 (Fig. 2A). As previously demonstrated, *max2* seed had significantly lower germination of approximately 5% and were insensitive to both KAR and SL treatment (Nelson et al., 2011). By contrast, both *smax1 max2* lines had high germination rates that matched or exceeded that of wild-type seed treated with KAR_2 or GR24. These results demonstrated that the *SMAX1* locus had been correctly identified and that *smax1* reverses the *max2* seed dormancy phenotype. We found that *smax1* also restores *max2* germination under high temperature conditions that induce secondary dormancy. *smax1 max2* seeds

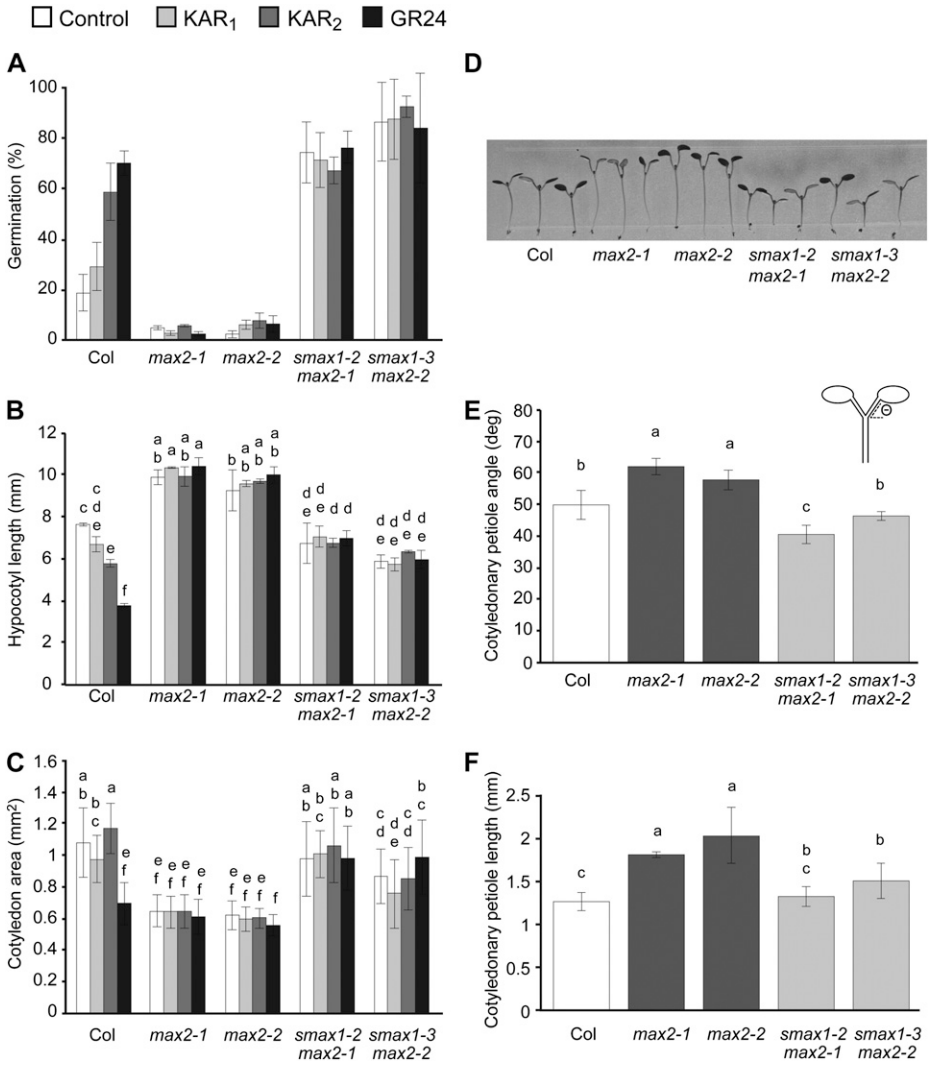

Figure 2. *smax1* suppresses seed and seedling stage phenotypes of *max2* mutants. The figure key applies to panels A, B, and C. A, The germination phenotype of *max2* is suppressed in *smax1 max2* mutants. Primary dormant seeds were grown on 0.8% agar plates containing 1 μM KAR_1, 1 μM KAR_2, or 10 μM GR24 for 5 d at 24°C. Mean ± SD (n = three experimental trials of 75–100 seeds per sample). B, *smax1* suppresses the elongated hypocotyl phenotype of 4-d-old *max2* seedlings grown in red light on 1 μM KAR_1, 1 μM KAR_2, or 1 μM GR24. Mean ± SE (n = two experimental trials of 20–50 hypocotyls per sample). Statistical groupings were determined by ANOVA with Tukey-Kramer HSD ($P < 0.01$). C, *smax1* suppresses the small cotyledon phenotype of 4-d-old *max2* seedlings grown in red light on 1 μM KAR_1, 1 μM KAR_2, or 1 μM GR24. Mean ± SD (n = 26–50 cotyledons per sample). Statistical groupings were determined by ANOVA with Tukey-Kramer HSD ($P < 0.01$). D, Enhanced contrast image of 7-d-old seedlings grown on soil in high humidity in 16-h white light/8-h dark. *smax1* suppresses cotyledonary petiole angle (E) and cotyledonary petiole length (F) of 7-d-old seedlings. Mean ± SE (n = three experimental trials of 26–40 petioles per sample). Statistical groupings were determined by ANOVA with Tukey-Kramer HSD ($P < 0.001$).

had higher germination rates than the wild-type and *max2* after a week of growth at 34°C followed by a week of growth at 24°C (Supplemental Fig. S2). High concentrations of GR24 produce a similar effect on wild-type seeds (Toh et al., 2012).

We next tested *smax1* for suppression of *max2* phenotypes during seedling development. *max2* seedlings have reduced growth responses to light, including elongated hypocotyls and smaller cotyledons (Stirnberg et al., 2002; Shen et al., 2007; Nelson et al., 2011). Hypocotyl elongation of *smax1 max2* was reduced to approximately 66% of the length of *max2*, at approximately 6 mm versus approximately 10 mm, respectively (Fig. 2B). *smax1 max2* hypocotyls were also significantly shorter ($P < 0.01$) than the wild-type grown on media lacking KAR/GR24 (approximately 8 mm) and were similar in length to wild-type seedlings treated with 1 μM KAR_1 or KAR_2 (approximately 6 mm). Cotyledons were also restored to wild-type size in *smax1 max2* seedlings (Fig. 2C). KAR promotes cotyledon expansion in L*er* seedlings, whereas GR24 has been shown to inhibit cotyledon expansion in both L*er* and Col-0 seedlings (Nelson et al., 2010; Waters et al., 2012b). Cotyledons of *smax1 max2* seedlings were more similar in size to control or KAR-treated Col-0 seedlings than GR24-treated Col-0 seedlings. Cotyledons of *smax1-1 max2-8* seedlings are substantially enlarged and also phenotypically similar to wild-type L*er* grown on KAR_2 (Supplemental Fig. S1).

The orientation of cotyledons is affected in *max2* seedlings, which have hyponastic and elongated petioles (Shen et al., 2007; Waters et al., 2012b). In seedlings grown for 7 d in soil under high humidity, the cotyledonary petiole angle of *max2* is fully restored by *smax1* (Fig. 2, D and E). The cotyledonary petioles of *smax1 max2* seedlings are also significantly shorter than those of *max2* seedlings (Fig. 2, D and F). Neither of these phenotypes differs between Col-0 and *smax1* single mutant seedlings (Supplemental Fig. S3, A and B).

These assays demonstrate that *smax1* suppresses an array of *max2* phenotypes at early stages of the plant life cycle. While *smax1* causes phenotypes that are

similar to KAR/SL treatment (e.g. rapid germination and modified photomorphogenesis), it does not replace the requirement for MAX2 in responses to KAR and SL (Fig. 2, A–C).

Expression of Three KAR/SL Transcriptional Markers Is Restored in *smax1 max2* Seedlings

Several transcriptional markers of KAR and SL response in seed and seedlings have previously been reported, including the genes *DLK2*, *KUF1*, and *SALT TOLERANCE HOMOLOG7* (*STH7*) (Nelson et al., 2010, 2011; Waters et al., 2012b). In addition to these KAR/SL-inducible markers, KAR/SL also represses the auxin-inducible transcriptional regulator *IAA1*. In *max2* seedlings, *DLK2*, *KUF1*, and *STH7* expression is repressed relative to wild-type controls, whereas *IAA1* transcripts are increased (Nelson et al., 2011; Waters et al., 2012b). We used qRT-PCR to assess the abundance of these markers in *smax1 max2* seedlings. In contrast to prior work, *STH7* expression was not significantly affected by KAR/SL treatment or the *max2* mutation, which could be an effect of ecotype or a subtle change in growth conditions (data not shown). However, in *smax1 max2* seedlings, *DLK2* and *IAA* transcripts were clearly restored to wild-type levels and *KUF1* transcripts even exceeded wild-type abundance (Fig. 3). Therefore, *smax1* suppresses the effects of *max2* on transcription of these markers of KAR and SL signaling. These observations are consistent with the hypothesis that SMAX1 acts downstream of MAX2 in the KAR/SL pathway.

Not All *max2* Phenotypes Are Suppressed by *smax1*

As there are distinct responses to KAR and SL, and *max2* has diverse developmental phenotypes, it is likely that multiple pathways are regulated by MAX2. We investigated whether SMAX1 is involved in the regulation of all such pathways, or only a subset, by assaying several additional *max2* phenotypes. Lateral root formation is increased in *max2* seedlings and suppressed by SL treatment (Kapulnik et al., 2011a). We found no significant difference in lateral root density between *smax1 max2* and *max2* seedlings (Fig. 4A). Next, we assessed the shoot architecture of 8-week-old *smax1 max2* plants. The number of axillary branches is increased, and the heights of the primary inflorescence stems are reduced in *max2* plants compared with the wild-type (Stirnberg et al., 2002). We found no significant difference in axillary branching or plant height between *smax1 max2* and *max2* (Fig. 4, B–D). For each of these assays, *smax1* single mutants resembled wild-type plants (Supplemental Fig. S3, C–E).

The *max2/ore9* mutant was first identified by its delayed leaf senescence phenotype (Woo et al., 2001). We observed no yellowing of *max2* or *smax1 max2* leaves grown in the dark for 6 d, whereas senescence

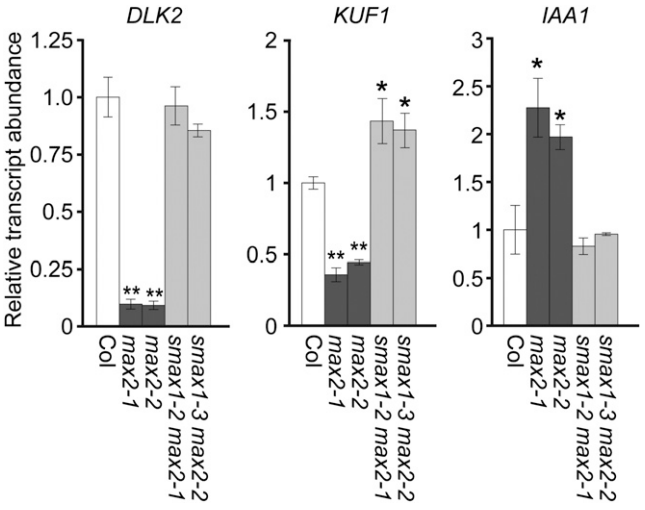

Figure 3. *smax1* restores expression of KAR/SL transcriptional markers in *max2* seedlings. Transcripts detected by real-time reverse transcription-PCR using complementary DNA derived from 4-d-old red-light-grown seedlings. Expression values are relative to the *CACS* reference gene and scaled to Col-0 = 1. Mean ± SE (n = three independent samples, >50 seedlings per sample). Significant difference from Col-0 was assessed by Student's t test (*$P < 0.025$, **$P < 0.001$).

was induced by this treatment in wild-type and *smax1* leaves (Fig. 4E). The leaf blades of *smax1 max2* plants were also not restored to a wild-type morphology (Supplemental Fig. S4). These data indicate that while SMAX1 influences seed germination and seedling growth, it is not involved in all aspects of MAX2-regulated growth. However, it is possible that functional redundancy could mask the role of SMAX1 in postseedling development.

SMAX1 Is a Member of an Eight-Gene Family with Similarity to AtHSP101

We used BLAST comparisons of the SMAX1 protein sequence to search for possible insights into its function. *SMAX1* is a member of an uncharacterized family of eight genes in Arabidopsis that are most closely related to *HEAT SHOCK PROTEIN 101* (*AtHSP101/HOT1*; Fig. 5). We hereafter refer to the homologs of *SMAX1* as *SMAX1-LIKE* (*SMXL*). *SMXL2* (*At4g30350*), *SMXL3* (*At3g52490*), *SMXL4* (*At4g29920*), *SMXL5* (*At5g57130*), *SMXL6* (*At1g07200*), *SMXL7* (*At2g29970*), and *SMXL8* (*At2g40130*) are predicted to encode proteins that share 27% to 57% identity with SMAX1. An N-terminal domain (approximately the first 163–193 amino acids) has the highest sequence conservation within this family, with 42% to 88% identity (Supplemental Fig. S5).

AtHSP101/HOT1 is a cytosolic Heat shock protein 100/Caseinolytic peptidase B (Hsp100/ClpB) protein that is necessary for tolerance to heat stress in plants (Hong and Vierling, 2000, 2001). Clp proteins are

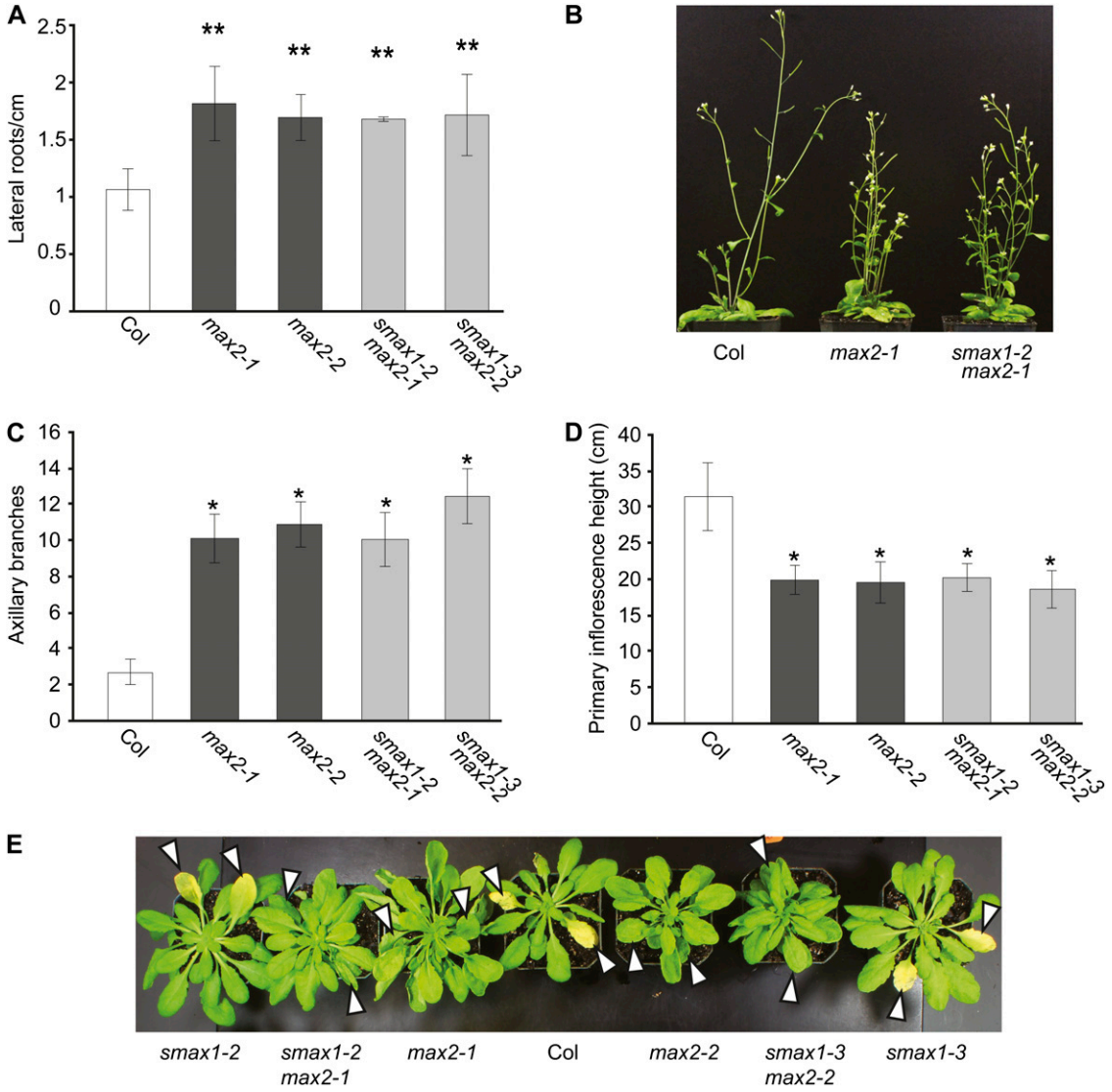

Figure 4. *smax1* does not suppress *max2* lateral root branching, axillary branching, primary inflorescence height, or dark-induced senescence phenotypes. A, Lateral roots per centimeter of primary root in 8-d-old seedlings. Mean ± SE (n = two experimental trials of 19–36 roots per sample). Significant differences compared with Col-0 were assessed by Student's t test (**$P < 1\times10^{-5}$). B, Thirty-nine-day-old plants. Axillary branching (C) and primary inflorescence height (D) phenotypes of 8-week-old *max2* plants. Mean ± SD (n = nine 8-week-old plants per genotype). Significant difference from Col-0 was assessed by Student's t test (*$P < 0.001$). E, Short-day-grown plants. Arrowheads indicate leaves kept in dark for 6 d to induce senescence.

molecular chaperones that prevent denaturation/aggregation of proteins, disassemble protein complexes, or solubilize protein aggregates, which are then fed into refolding or proteolytic machinery (Schirmer et al., 1996). AtHSP101 is 45% identical to its ortholog in yeast (*Saccharomyces cerevisiae*), ScHsp104. AtHSP101 has similar function to ScHsp104, as both are induced by heat stress and expression of AtHSP101 in yeast rescues the thermotolerance defect of the Δ*Hsp104* mutant (Schirmer et al., 1994). By contrast, AtHSP101 and SMAX1 are only 18% identical. It remains to be determined whether SMAX1 and SMXL proteins have ClpB-like chaperonin activity.

SMAX Family Genes Are Differentially Expressed during Development

To determine if *SMAX1* gene expression is consistent with its developmental roles, we investigated the abundance of *SMAX1* transcripts in several plant tissues in which *max2* phenotypes have been observed, including seeds, seedlings, roots, green leaves, senescent leaves, and axillary stems. For comparison, we also tested the expression of the *SMXL* genes (Fig. 6).

SMAX1 transcripts were particularly high in dry seed compared with the other tissues. In seed, *SMAX1* transcripts were also substantially more abundant

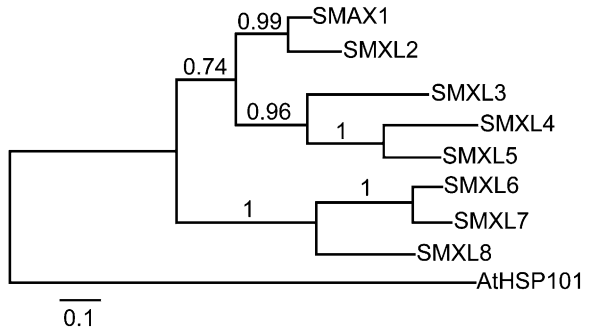

Figure 5. Maximum-likelihood phylogram of SMAX1 and SMAX1-like proteins in Arabidopsis. SMAX1, SMXL2 (At4g30350), SMXL3 (At3g52490), SMXL4 (At4g29920), SMXL5 (At5g57130), SMXL6 (At1g07200), SMXL7 (At2g29970), SMXL8 (At2g40130), and HSP101 were assigned to the tree by PhyML based on protein sequence similarity. Numbers above the branches represent bootstrap support derived from 100 bootstrap replicates. Scale bar (branch length) represents substitutions per site.

than *SMXL* transcripts. Publicly available microarray data suggest that *SMAX1* expression peaks in seeds during maturation (Supplemental Fig. S6; Schmid et al., 2005; Winter et al., 2007). This expression pattern is consistent with a role for SMAX1 in seed germination or dormancy.

SMAX1 was expressed more highly than *SMXL* genes in seedlings and rosette leaves and was expressed comparably to *SMXL7* in senescing leaves. In roots, the expression of this gene family was generally low, and *SMXL3* was predominant. In axillary shoots, *SMXL7* transcript was most abundant. *SMXL4* and *SMXL8* transcripts were typically present in these tissues at the lowest abundance of this gene family. If *SMXL* genes have similar function to *SMAX1*, these tissue-specific expression patterns might reflect their respective roles in MAX2-regulated development.

Transcriptional Responses to KAR/SL in the *SMXL* Family

SMAX1 and its closest homolog, *SMXL2*, were previously identified as KAR-induced genes in primary dormant L*er* or *ga requiring1-3* (*ga1-3*) mutant seed imbibed for 24 h on KAR_1 (Nelson et al., 2010). Similarly, *SMAX1*, *SMXL2*, and *SMXL7* were among the 31 genes reported as transcriptionally induced in *max3* seedlings by a 90-min GR24 treatment (Mashiguchi et al., 2009). In both experiments, the transcriptional response of these genes to KAR_1 or GR24 was modest, at approximately 1.5- to 2-fold.

We tested the transcriptional responses of *SMAX1* and *SMXL* genes to KAR_2 and GR24 in 4-d-old Arabidopsis seedlings grown in red light. Several genes, including *SMXL2*, *SMXL3*, *SMXL6*, *SMXL7*, and *SMXL8*, were up-regulated by GR24 treatment ($P < 0.05$). In this experiment, however, only *SMXL2* and *SMXL8* were significantly induced by KAR_2 treatment ($P < 0.05$). To confirm that the KAR_2 treatment was effective, we also tested *DLK2* expression. *DLK2* transcripts were induced in KAR_2-treated seedlings, albeit less strongly than in GR24-treated seedlings (Fig. 7A).

As a complementary approach to investigate the regulation of *SMAX1* and *SMXL* genes by the KAR/SL pathway, we assayed the transcript abundance of this family in the *max2* mutant. *SMAX1* and *SMXL2* transcripts were reduced by approximately 30% in *max2* seedlings (Fig. 7B). Other *SMXL* transcripts were not significantly affected (data not shown). The reduction of *SMAX1* transcripts in *max2-1* seedlings is consistent with a hypothesis of MAX2-dependent negative feedback regulation on *SMAX1* expression. The observations that *SMXL2* is also down-regulated in *max2-1* and that several *SMXL* genes are induced by GR24 strengthen the idea that some *SMXL* genes may have similar roles to *SMAX1* as suppressors of KAR/SL responses.

SMAX1 Function Is Partially Redundant in Seedlings

We reasoned that if *SMAX1* has a nonredundant role downstream of *MAX2* in a given aspect of development, the *smax1* mutant would constitutively mimic KAR/SL responses and would also be insensitive to KAR/SL treatment. To test this hypothesis, we examined *smax1* responses during seedling growth, a developmental stage in which SMAX1 clearly acts. *DLK2* and *KUF1* transcripts were induced in *smax1* seedlings, as has previously been observed for wild-type treated with KAR/SL (Fig. 8A; (Waters et al., 2012b). Consistent with the assays of *smax1-2 max2-1* seedlings (Fig. 2), *smax1-2* grown in the absence of KAR/GR24 had similar morphology to KAR-treated wild-type seedlings (Fig. 8, A–C). However, hypocotyl elongation and cotyledon expansion of *smax1-2* seedlings was weakly responsive to KAR_2 and clearly responsive to GR24 treatment (Fig. 8, B and C). Therefore, other genetic components must be involved in SL responsiveness in seedlings. The *SMXL* genes that are moderately expressed in seedlings, *SMXL2*, *SMXL3*, *SMXL5*, *SMXL6*, and *SMXL7*, are potential candidates for this functional redundancy (Fig. 6). It is unclear if there could be similar redundancy with SMAX1 during seed germination, as the rapid germination rate of *smax1-2* seeds made it difficult to detect positive responses to KAR or GR24 treatment (Fig. 8D).

DISCUSSION

A series of genes that act upstream of *MAX2* in the KAR/SL pathway in Arabidopsis have been defined, including the SL biosynthetic enzymes *MAX3/CAROTENOID CLEAVAGE DIOXYGENASE7* (*CCD7*), *MAX4/CCD8*, *MAX1*, and *D27* (Alder et al., 2012; Waters et al., 2012a), as well as the candidate receptors *KAI2* and *D14* (Hamiaux et al., 2012; Waters et al., 2012b). Several molecular outputs of the KAR/SL pathway have been described, including transcriptional responses (Nelson

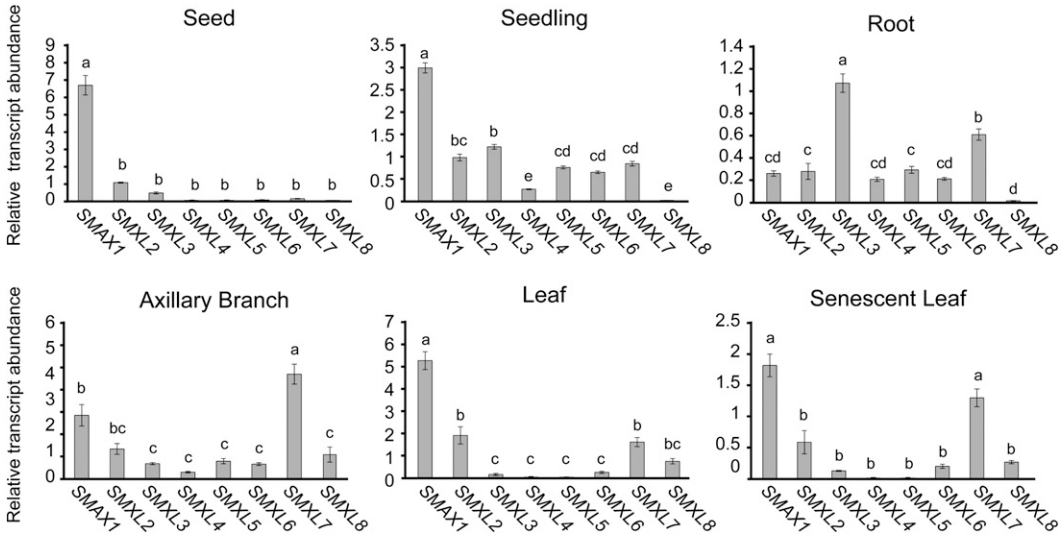

Figure 6. Relative abundance of *SMAX1* and *SMXL* transcripts in Arabidopsis tissues. Transcripts detected by real-time reverse transcription-PCR using complementary DNA derived from the following tissues from wild-type plants: dry seed (n = four samples, approximately 40 mg per sample), 4-d-old red-light-grown seedlings (n = five samples, >50 seedlings per sample), 13-d-old roots (n = four samples, >50 roots per sample), 5-cm-long sections from axillary branches on 6-week-old plants, each section containing three axillary buds and cauline leaves (n = four samples, one section per sample), 2-cm-long rosette leaves from 6-week-old plants (n = four samples, two leaves per sample), and senescent rosette leaves with approximately 50% remaining green from 7-week-old plants (n = four samples, one leaf per sample). Abundance values are relative to the *CACS* reference gene. Mean ± SE. Statistical groupings for each tissue type were determined by ANOVA with Tukey-Kramer HSD ($P < 0.01$).

et al., 2010, 2011; Waters et al., 2012b) and effects on PIN-FORMED1 distribution (Crawford et al., 2010; Shinohara et al., 2013), but the signal transduction steps downstream of MAX2 are unknown. To address this problem, we undertook a forward genetic screen for *smax* that have recovered rapid seed germination. Recognizing that mutations in genes that are not specific to the MAX2 pathway (e.g. components of the abscisic acid or gibberellic acid pathways) could easily confound a screen based on germination alone, we filtered putative *smax* mutants by testing for suppression of multiple *max2* phenotypes. *smax1-1* was chosen for further study, mapped, and identified as a premature stop codon in the previously uncharacterized gene *At5g57710* (Fig. 1A).

Mutant alleles of *SMAX1* restored several *max2* phenotypes in seed germination and seedling growth (Figs. 2 and 3). While *max2* seeds germinate at a very low rate, *smax1 max2* seeds germinate with even greater frequency than the wild-type. The longer hypocotyls and smaller cotyledons seen in red light-grown *max2* seedlings were suppressed. The increased length and upward orientation of cotyledonary petioles of *max2* were also suppressed in *smax1 max2* seedlings. Altered expression of three transcriptional markers of KAR/SL response in *max2* seedlings was restored by *smax1* (Fig. 3). However, *smax1* did not affect axillary shoot branching, primary inflorescence height, lateral root density, or dark-induced senescence (Fig. 4; Supplemental Fig. S3).

Altogether, these experiments demonstrate that SMAX1 contributes to MAX2-related physiological processes during early growth phases but is not required at other developmental stages. It remains possible that SMAX1 is a component of a parallel pathway and is not specific to the MAX2 pathway. The *smax1* single mutant has low seed dormancy and short hypocotyls (Fig. 8); therefore, it could indirectly counteract *max2* phenotypes and only coincidentally mimic constitutive KAR responses. However, we do not favor this idea for two reasons. First, *smax1* restores the expression of three markers of KAR/SL signaling as well as five developmental phenotypes in *max2*. It would be highly remarkable for a parallel pathway to affect all of these processes at a molecular and morphological level in the same way as the MAX2 pathway. Moreover, cotyledonary petiole growth (Supplemental Fig. S3, A and B) and *IAA1* expression (Fig. 8A) were not significantly affected in *smax1* seedlings but were restored to wild-type phenotypes in *smax1 max2* (Figs. 2 and 3); therefore, overcompensation by *smax1* was not a factor in *max2* suppression. Second, *SMAX1* and *SMXL2* transcripts are down-regulated in *max2*, and several *SMXL* homologs are transcriptionally responsive to KAR/GR24 (Fig. 7). If SMAX1 acts in a parallel pathway, this would imply complex transcriptional crosstalk with the MAX2 pathway. We therefore propose the simpler explanation that SMAX1 acts downstream of MAX2 to negatively regulate KAR/SL responses.

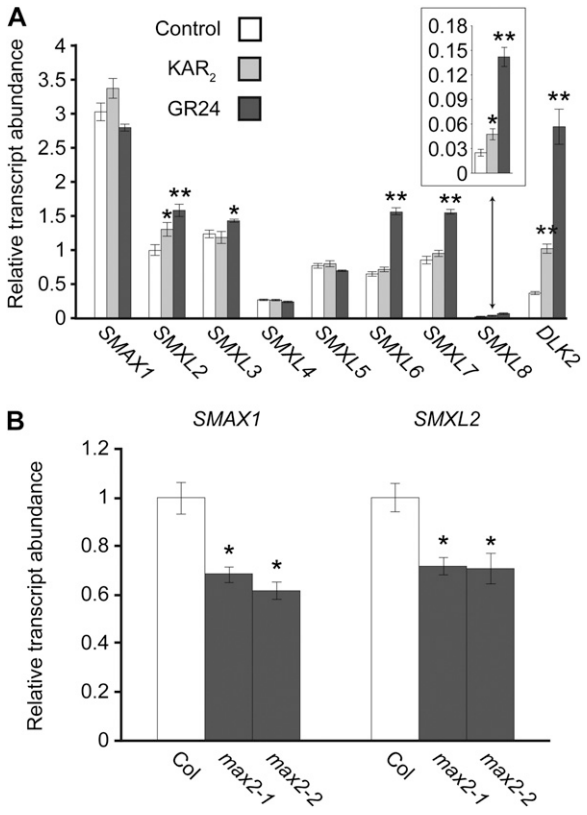

Figure 7. Transcriptional responses to KAR/SL in the *SMXL* gene family. A, *SMAX1* and *SMXL* transcriptional responses to growth on 1 μM KAR2 or 1 μM GR24 in 4-d-old red-light-grown seedlings. *DLK2* included as a positive control for KAR_2 and GR24 response. Transcripts detected by real-time reverse transcription-PCR. Expression values are relative to the *CACS* reference gene. Mean ± SE (n = five independent samples, >50 seedlings per sample). Significant differences compared with the control treatment were assessed by Student's t test (*$P < 0.05$, **$P < 0.01$). B, *SMAX1* and *SMXL2* transcripts in 4-d-old red-light-grown *max2* seedlings. Transcripts detected by real-time reverse transcription-PCR, and values are relative to *CACS* and scaled to Col-0 = 1. Mean ± SE (n = three independent samples, >50 seedlings per sample). Significant differences to Col-0 were assessed by Student's t test (*$P < 0.05$).

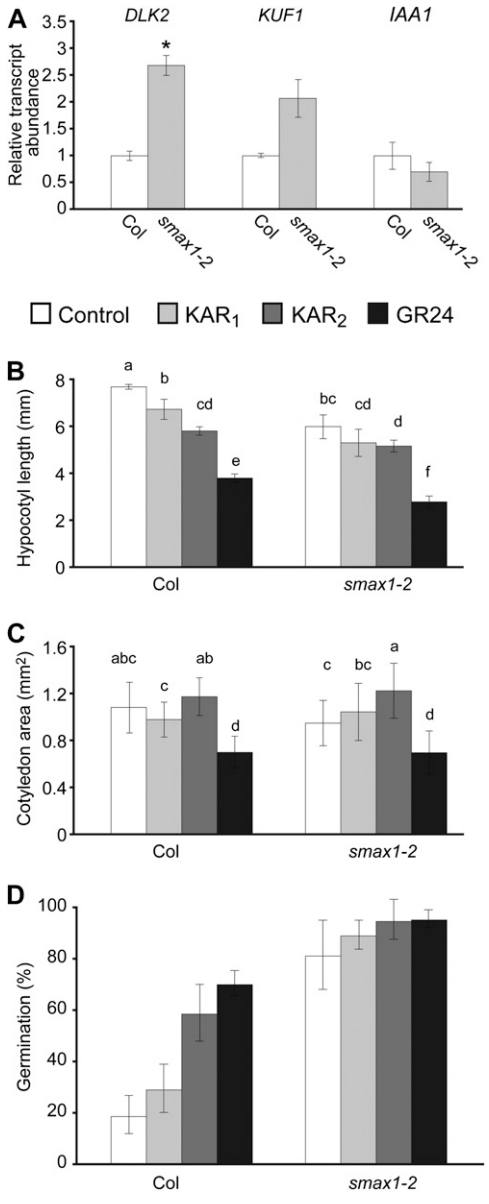

Figure 8. *smax1* single mutant phenotypes. The figure key applies to B, C, and D. A, Relative abundance of KAR/SL-responsive transcripts detected by real-time reverse transcription-PCR using complementary DNA derived from 4-d-old red-light-grown seedlings. Values are relative to the *CACS* reference gene and scaled to Col-0 = 1. Mean ± SE (n = three independent samples, >50 seedlings per sample). Significant difference from Col-0 was assessed by Student's t test (*$P < 0.05$). B, Hypocotyl lengths of 4-d-old seedlings grown in red light on 1 μM KAR_1, 1 μM KAR_2, or 1 μM GR24. Mean ± SE (n = two experimental trials of 20–50 hypocotyls per sample). Statistical groupings were determined by ANOVA with Tukey-Kramer HSD ($P < 0.01$). C, Cotyledon area of 4-d-old seedlings grown in red light on 1 μM KAR_1, 1 μM KAR_2, or 1 μM GR24. Mean ± SD (n = 32–50 cotyledons per sample). Statistical groupings were determined by ANOVA with Tukey-Kramer HSD ($P < 0.01$). D, Primary dormant seeds were grown on 0.8% agar plates containing 1 μM KAR_1, 1 μM KAR_2, or 10 μM GR24 for 5 d at 24°C. Mean ± SD (n = three experimental trials of 75–100 seeds per sample).

SMAX1 has seven *SMXL* homologs in Arabidopsis (Fig. 5). We hypothesize that one or more of these homologs also have roles in developmental control downstream of MAX2. Functional redundancy within this gene family could explain why *smax1* does not suppress all *max2* phenotypes and why these genes have not been reported from prior *max2* suppressor screens that were based upon axillary branching or leaf senescence phenotypes. Our approach of testing for suppression of *max2* phenotypes at early stages of development may have provided an entry point into the downstream pathway where, fortuitously, redundancy among *SMXL* genes is minimal.

We characterized the expression of *SMAX1* and *SMXL* genes in several plant tissues (Fig. 6). The high expression of *SMAX1* in seeds and seedlings is consistent

with its function at these stages. However, while *SMAX1* is also highly expressed in leaves relative to *SMXL* genes, *smax1* did not restore normal leaf shape to *max2* rosettes. Therefore, in some, but not all, cases, tissue-specific gene expression patterns may provide clues to function for this family. For example, *SMXL3* is expressed approximately 5-fold higher than *SMAX1* in roots, making it a candidate for having a role in root growth. Similarly, as *SMXL7* is relatively highly expressed in axillary stem tissue, it is a good candidate for control of axillary branching. It is notable that several *SMXL* genes were induced by GR24 treatment, as this supports a connection between the SMXL family and SL signaling (Fig. 7). Higher order genetic analyses of mutants in this family will be required to eliminate potential redundancy and determine if the SMXL proteins act downstream of MAX2.

What Is the Role of SMAX1 in the KAR/SL Pathway?

F-box proteins such as MAX2 have been implicated in the direct perception or early signal transduction of several plant hormones, including auxins, jasmonates, ethylene, and gibberellins (GAs; Somers and Fujiwara, 2009). In the GA signaling mechanism, the GA INSENSITIVE DWARF1 receptors undergo a conformational change after binding GA that stimulates interaction with a class of repressor proteins known as DELLAs (for review, see Sun, 2011). The five DELLA proteins in Arabidopsis (GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GA (RGA), RGA-LIKE1 (RGL1), RGL2, and RGL3) control developmental responses to GA in a partially redundant manner; for example, RGL2 has a prominent role as a repressor of seed germination, whereas RGA and GAI are key repressors of stem elongation (Dill and Sun, 2001; Cao et al., 2005). Recognition of the GA-GA INSENSITIVE DWARF1-DELLA complex by the F-box protein SLEEPY1 leads to polyubiquitination and degradation of DELLA and the relief of GA pathway repression (Sun 2011).

There are remarkable similarities between the KAR/SL and GA signaling pathways; like the GA receptors, KAI2 and D14 are classified as α/β-hydrolases, and an F-box protein is required in both mechanisms. These similarities have led to the hypothesis that the target(s) of MAX2 are analogous to DELLAs in their roles and regulation (Nelson et al., 2011; Waters et al., 2012b). This idea has two important implications. The first is that the substrates of MAX2 are KAR/SL pathway repressors that are targeted for degradation following perception of KAR/SL. The second is that MAX2, which controls several developmental processes, may be expected to have multiple homologous targets. Removal of all targets of MAX2 in a wild-type MAX2 background would therefore be expected to confer constitutive KAR/SL responses, as well as insensitivity to KAR/SL treatment.

Could SMAX1 be the repressor substrate of MAX2? Although *smax1* mutants have phenotypes that mimic KAR/SL treatment (e.g. rapid germination, enhanced seedling photomorphogenesis, and up-regulation of *DLK2* and *KUF1*), *smax1* is still responsive to KAR/SL treatment (Fig. 8). Therefore, if SMAX1 is targeted by MAX2, it is not the only target, even at early stages of growth. One alternative hypothesis for SMAX1 function is that it promotes the activity of a repressor protein that is targeted for degradation by MAX2 following KAR/SL perception.

SMAX1 has weak similarity to the ClpB protein HSP101/HOT1. ClpB proteins are typically involved in disaggregating proteins, particularly in response to stress (Parsell et al., 1994; Schirmer et al., 1996). AtHSP101, the cytoplasmic form of ClpB in Arabidopsis, is required for thermotolerance (Hong and Vierling, 2000, 2001). Interestingly, in two cases, HSP101 has also been implicated in translational regulation. HSP101 binds the 5′ leader sequence (Ω) of tobacco mosaic virus, leading to its enhanced translation (Wells et al., 1998). Similarly, HSP101 binds the 5′ leader component of the internal light regulatory element of ferredoxin mRNA and thereby promotes translational activity (Ling et al., 2000).

Transcriptional responses to KAR and SL are modest in terms of the number of genes affected and the magnitude of fold changes, whether after 90-min or 24-h treatments (Mashiguchi et al., 2009; Nelson et al., 2010). It was recently demonstrated that depletion of the auxin efflux carrier PIN-FORMED1 from the plasma membrane occurs within 10 min after SL treatment and does not depend upon protein synthesis (Shinohara et al., 2013). As such, it is the earliest known response to SL. This suggests that a posttranslational regulatory mechanism, rather than a transcriptional cascade, may carry out the initial responses to KAR/SL perception. It remains to be determined how SMAX1 contributes to KAR/SL signaling. If SMAX1 has retained ClpB-like activity, it may disassemble specific protein complexes/aggregates or regulate translation of specific mRNAs.

MATERIALS AND METHODS

Genetic Screen for Suppressors of *max2*

max2-8 seeds were mutagenized with 0.25% (v/v) ethyl methane sulfonate according to Weigel and Glazebrook (2002). Approximately 2,500 M_1 plants were grown in continuous light at 22°C and harvested in 39 pools. Approximately 50,000 primary dormant M_2 seeds were screened on 0.5× Murashige and Skoog (MS) basal salt mixture plus 1 μM 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (KAR_1), and another 50,000 primary dormant M_2 seeds were screened on 0.8% (w/v) agar plus 1 μM 2*H*-furo[2,3-*c*]pyran-2-one (KAR_2) for germination after 3 d growth at 20°C in continuous white light. Early germinants with reduced hypocotyl elongation or recovered cotyledon morphology were preferentially selected. Primary dormant M_3 seeds from 286 putative suppressor mutants were rescreened for rapid germination on 0.8% (w/v) Bacto-agar plus 1 μM KAR_2. Fourteen rapidly germinating M_3 lines were tested with a red-light photomorphogenesis assay. Under these conditions, *smax1-1 max2-8* seedlings had short hypocotyls and expanded cotyledons that were morphologically similar to wild-type L*er* seedlings grown on 1 μM KAR_2.

KAR_1, KAR_2, and GR24 were synthesized by Adrian Scaffidi (University of Western Australia). 1000X stocks in acetone were prepared for each compound and stored at –20°C.

Germination Assay

Seedlings were grown on 0.5× MS plates, transferred to soil after approximately 10 to 15 d, and grown under fluorescent light (approximately

80–110 μmol m^{-2} s^{-1}) with 16-h-light/8-h-dark cycles at 21°C. Pots were randomized within each flat to minimize effects from environmental variation. Plants were harvested when most siliques were brown and dried in paper bags at room temperature for 3 d. Seeds were cleaned, equilibrated in a box containing Drierite desiccant for 3 d, and stored at –80°C to preserve dormancy. Before assays, seeds were surface sterilized with a 70% (v/v) ethanol and 0.05% (v/v) Triton X-100 solution for 5 min, rinsed with 70% (v/v) and 95% (v/v) ethanol, and dried on filter paper. Seeds were sprinkled onto 0.8% (w/v) Bacto-agar supplemented with either 1 μM KAR_1, 1 μM KAR_2, 10 μM GR24, or an acetone control (final concentration, 0.1% [v/v]). Plates were incubated at 24°C under constant white light (approximately 50–70 μmol m^{-2} s^{-1}). Germination was indicated by emergence of the radicle tip through the endosperm. Independent seed batches grown under similar conditions were assessed.

Thermoinduced Secondary Dormancy Assay

Thermoinduced secondary dormancy assays were performed similarly to Toh et al. (2012). Seeds were harvested and stored at ambient conditions for 8 months. Seeds were sprinkled onto 0.8% (w/v) Bacto-agar supplemented with either 1 μM KAR_2, 10 μM GR24, or an acetone control (final concentration, 0.1% [v/v]). Plates were incubated for 7 d at 34°C under constant white light (approximately 50–70 μmol m^{-2} s^{-1}) and then for 7 d at 24°C. Germination was indicated by emergence of the radicle tip through the endosperm.

Seedling Photomorphogenesis Assay

Sterilized seeds were plated on solid 0.5× MS medium supplemented with 1 μM KAR_1, 1 μM KAR_2, 1 μM GR24, or an acetone control and then stratified for 3 d at 4°C in the dark. Seeds were exposed to 3-h white light (approximately 40–80 μmol m^{-2} s^{-1}) at 21°C, returned to dark for 21 h at 21°C, and then horizontally grown for 4 d at 24°C under continuous red light (approximately 10 μmol m^{-2} s^{-1}) provided by light-emitting diode strip lights (EagleLight). Plates were photographed, and the cotyledons were manually removed and laid flat to be photographed. Hypocotyl lengths and cotyledon areas were measured using ImageJ (http://rsb.info.nih.gov/ij/).

Lateral Root Assay

Lateral root assays were performed according to Koren et al. (2013). Sterilized seeds were plated onto 0.5× MS medium containing 1% (w/v) Bacto-agar and 1.5% (w/v) Suc and stratified for 3 d at 4°C in the dark. Plates were left unsealed, tilted back at a 45° angle, and grown for 8 d at 21°C and approximately 40 to 80 μmol m^{-2} s^{-1} light under 16-h-light/8-h-dark cycles. Lateral root emergence was observed under a stereomicroscope and scored from the stage of emergence (Malamy and Benfey, 1997). Plates were then photographed, and root lengths were measured using ImageJ.

Dark-Induced Senescence Assay

Dark-induced senescence assay was modified from Weaver and Amasino (2001) and Keech et al. (2007). Plants were grown under fluorescent light (approximately 160–220 μmol m^{-2} s^{-1}) with 8-h-light/16-h-dark cycles at 21°C for 5 weeks. Two leaves per plant were covered with aluminum foil for 6 d and then photographed.

Genotyping

To genotype *max2-1*, primers (listed in Supplemental Table S1) were designed based on the output from dCAPS Finder (Neff et al., 2002). PCR was performed with the following program: 2 min at 96°C; 35 cycles of 10 s at 96°C, 15 s at 54°C, and 15 s at 72°C; 1 min at 72°C. PCR products were digested with *Eco*RI; the *max2-1* PCR fragment is cut and the wild-type remains uncut. Other genotyping was performed with the primers listed in Supplemental Table S1 and standard PCR conditions.

qRT-PCR

Total RNA was isolated from Arabidopsis (*Arabidopsis thaliana*) tissues (dry seeds, 5-d red-light-grown seedlings, 12-d-old roots, green rosette leaves, 5-cm axillary stems containing floral buds and young cauline leaves, and senescing rosette leaves with approximately 50% green tissue) using a Spectrum Plant Total RNA Kit (Sigma). RNA was DNAse treated with Turbo DNA-Free (Ambion) and converted to complementary DNA with iScript Reverse Transcription Supermix that uses random hexamer and dT primers (Bio-Rad). Complementary DNAs were used as template for quantitative PCR in a Roche Light Cycler 480 using LightCycler 480 SYBR Green I Master (Roche) with the following program: 10 min at 95°C and 45 cycles of 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C, followed by melt curve analysis to analyze product specificity. Most primer pairs were designed using AtRTPrimer (Han and Kim, 2006), and all primer pairs used for *SMXL* expression analyses span introns, except for *SMXL7*. *CACS*, *DLK2*, *KUF1*, and *IAA1* primer sequences are from Nelson et al. (2011) and Waters et al. (2012b). Primer sequences are listed in Supplemental Table S1. Crossing point values were calculated under high confidence with Light Cycler 480 software. The average crossing point of two technical replicates was to calculate the abundance relative to the *CACS* reference gene for each sample (Nelson et al., 2009), with adjustment for primer efficiencies. Relative expression was calculated as $(1 + Eff_{Ref})^{CpRef}/(1 + Eff_{Exp})^{CpExp}$ where Eff_{Ref} = reference primer efficiency, CpRef = reference crossing point value, Eff_{Exp} = experimental primer efficiency, and CpExp = experimental crossing point value (Pfaffl, 2001). Relative expression from at least three biological samples was averaged for each data point.

Protein Sequence Analysis

PhyML 3.0 (Guindon et al., 2010) was used to construct a maximum-likelihood phylogram from full-length protein sequences obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org). The default substitution model was used with 100 bootstraps. Protein alignments were created using the ClustalW algorithm of MegAlign software (DNASTAR).

Statistical Tests

Statistics were calculated using the JMP statistical package (SAS) or Excel (Microsoft). Prior to Student's *t* tests, data were tested for equal variance by F-tests. One-way ANOVA tests were conducted using combined data from experimental replicates. Following ANOVA, Tukey-Kramer honestly significant difference (HSD) post hoc tests were conducted to assign statistical groupings. All sample sizes and significance thresholds are indicated in the figure legends.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *smax1 max2* seedlings.

Supplemental Figure S2. Thermoinduced secondary dormancy.

Supplemental Figure S3. *smax1* phenotypes.

Supplemental Figure S4. *smax1 max2* leaf morphology.

Supplemental Figure S5. Alignment of SMAX1 and SMXL N-termini.

Supplemental Figure S6. *SMAX1* expression in seeds.

Supplemental Table S1. Primers used in this study.

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