Strigolactone- and Karrikin-Independent SMXL Proteins Are Central Regulators of Phloem Formation

Highlights
- SMXL3/4/5 genes act as general promoters of phloem formation
- SMXL3/4/5 proteins are expressed and function very early during phloem development
- SMXL3/4/5 proteins do not mediate strigolactone or karrikin signaling
- Strigolactone/karrikin-dependent SMXL proteins are able to replace SMXL5

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In Brief
Plants depend on long-distance transport of energy metabolites and signaling molecules along the phloem tissue. Wallner et al. show that phloem formation requires a family of proteins closely related to mediators of a hormonal signalling pathway. An hormone-independent action of the proteins analyzed is essential for robust phloem formation.
Strigolactone- and Karrikin-Independent SMXL Proteins Are Central Regulators of Phloem Formation

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SUMMARY

Plant stem cell niches, the meristems, require long-distance transport of energy metabolites and signaling molecules along the phloem tissue. However, currently it is unclear how specification of phloem cells is controlled. Here we show that the genes SUPPRESSOR OF MAX2 1-LIKE3 (SMXL3), SMXL4, and SMXL5 act as cell-autonomous key regulators of phloem formation in Arabidopsis thaliana. The three genes form an uncharacterized subclade of the SMXL gene family that mediates hormonal strigolactone and karrikin signaling. Strigolactones are endogenous signaling molecules regulating shoot and root branching [1] whereas exogenous karrikin molecules induce germination after wildfires [2]. Both activities depend on the F-box protein and SCF (Skp, Cullin, F-box) complex component MORE AXILLARY GROWTH2 (MAX2) [3–6]. Strigolactone and karrikin perception leads to MAX2-dependent degradation of distinct SMXL protein family members, which is key for mediating hormonal effects [6–12]. However, the nature of events immediately downstream of SMXL protein degradation and whether all SMXL proteins mediate strigolactone or karrikin signaling is unknown. In this study we demonstrate that, within the SMXL gene family, specifically SMXL3/4/5 deficiency results in strong defects in phloem formation, altered sugar accumulation, and seedling lethality. By comparing protein stabilities, we show that SMXL3/4/5 proteins function differently to canonical strigolactone and karrikin signaling mediators, although being functionally interchangeable with those under low strigolactone/karrikin signaling conditions. Our observations reveal a fundamental mechanism of phloem formation and indicate that diversity of SMXL protein functions is essential for a steady fuelling of plant meristems.

RESULTS

SMXL3, SMXL4, and SMXL5 Act on Primary Root Growth
To reveal the function of the uncharacterized SMXL sub-clade 2 (Figure 1A), we phenotypically characterized single mutant seedlings as well as all possible double mutant combinations and the smxl3;smxl4;smxl5 triple mutant (Figures S1A and S1B). In contrast to single mutants, which displayed no obvious phenotypic alterations to wild-type, primary root length was substantially reduced in all double mutants (Figures 1B and 1C), suggesting a redundant and equal contribution of all three genes to primary root growth. These defects were not observed in smxl1;smxl2 double or smxl6;smxl7;smxl8 triple mutants, defective for SMXL sub-clades 1 and 3, respectively (Figure 1D). Strikingly, smxl3;smxl4;smxl5 triple mutant seedlings were lethal (Figure 1E), while resembling double mutants at early growth stages (Figures 1B and 1F). This indicated a unique and dose-dependent role of the SMXL3, SMXL4, and SMXL5 genes in growth regulation.

SMXL3, SMXL4, and SMXL5 Are Expressed in Phloem-Associated Tissues
To see whether SMXL3, SMXL4, and SMXL5 share similar expression patterns supporting functional redundancy, we stably expressed an endoplasmic reticulum (ER)-localized YELLOW FLUORESCENT PROTEIN (YFP) under the control of the respective promoters. As a reference we confirmed promoter activity of the karrikin (KAR) mediator SMAX1 in the vasculature and columella (Figures S1C–S1E) [10, 13, 14]. SMXL3 promoter activity was predominantly found in the root vasculature and weakly in the vasculature of cotyledons (Figures S1F–S1H). SMXL4 and SMXL5 promoter activities were specific for vascular tissues in all organs analyzed with a particular strong activity in root tips (Figures S1I–S1N), which was consistent with earlier findings for SMXL4 [15, 16]. Close inspection of the root apical meristem
(RAM) revealed activities of all promoters immediately proximal to the quiescent center (QC) in sieve element (SE)-procambium stem cells and maturating phloem poles (Figure 1G). This finding was confirmed in cross sections that revealed SMXL4 promoter activity in the two phloem poles and SMXL3 and SMXL5 promoter activities in both the developing phloem and the procambium (Figures 1H and 1I). In comparison, the SMAX1 promoter was only weakly active in phloem-associated regions (Figures 1G–1I). Taking these observations together, we concluded that, in the RAM, SMXL3, SMXL4, and SMXL5 promoters are specifically active in phloem-related tissues.

**SMXL3, SMXL4, and SMXL5 Promote Phloem Formation**

To specify the function of SMXL sub-clade 2 members in root growth, we examined RAM anatomy of smxl4;smxl5 mutants. Interestingly, the RAM size in smxl4;smxl5 plants was indistinguishable from wild-type in 2-day-old seedlings, but decreased progressively over a period of 10 days (Figures 2A–2G). This suggested that maintaining RAM activity requires SMXL4/5 gene functions. Due to their phloem-associated expression and because phloem defects result in similar alterations in RAM performance [18, 19], we predicted a role of the SMXL sub-clade 2 in phloem...
formation. To test this, we investigated protophloem differentiation in 2-day-old seedlings when the **smxl4;smxl5** RAM size and the total number of stele cells was still unaffected (Figures 2G and S1O–S1Q). Indeed, **smxl4;smxl5** plants showed a delay in the second tangential cell division giving rise to proto- and metaphloem strands (Figures 2H–2K) [20]. Likewise, **smxl4;smxl5** RAMs were devoid of enhanced propidium iodide (PI) staining indicating differentiated SEs [21] (Figures 2I, 2J, S1O, and S1P). Reconstructions of 3D representations from ultrathin sections generated through serial block face scanning electron microscopy (SBEM) [22] confirmed that protophloem differentiation was substantially impaired in **smxl4;smxl5** plants. Cytosol density stayed high in cells located at positions expected for developing SEs (Figure 3A). In particular, enucleation, one of the first signs of SE differentiation in wild-type, was absent in **smxl4;smxl5** plants [24] (Figures 3B–3E). Moreover, activity of a CALLOSE SYNTHASE7:H2B-YFP (CALS7:H2B-YFP) marker visualizing nuclei of early protophloem cells prior to enucleation [24] was absent in **smxl4;smxl5** roots (Figures 3F and 3G). Taking these observations together, we concluded that early processes during SE formation are impaired in **smxl4;smxl5** RAMs.

To demonstrate a context-independent role of **SMXL4/5** in phloem formation, we investigated phloem differentiation capacity of **smxl5** and **smxl4;smxl5** cotyledons employing VISUAL [23, 25]. Before trans-differentiation, phloem appearance was comparable between wild-type and **smxl5** and **smxl4;smxl5** plants. To test this, we investigated protophloem differentiation in 2-day-old seedlings when the **smxl4;smxl5** RAM size and the total number of stele cells was still unaffected (Figures 2G and S1O–S1Q). Indeed, **smxl4;smxl5** plants showed a delay in the second tangential cell division giving rise to proto- and metaphloem strands (Figures 2H–2K) [20]. Likewise, **smxl4;smxl5** RAMs were devoid of enhanced propidium iodide (PI) staining indicating differentiated SEs [21] (Figures 2I, 2J, S1O, and S1P). Reconstructions of 3D representations from ultrathin sections generated through serial block face scanning electron microscopy (SBEM) [22] confirmed that protophloem differentiation was substantially impaired in **smxl4;smxl5** plants. Cytosol density stayed high in cells located at positions expected for developing SEs (Figure 3A). In particular, enucleation, one of the first signs of SE differentiation in wild-type, was absent in **smxl4;smxl5** plants [24] (Figures 3B–3E). Moreover, activity of a CALLOSE SYNTHASE7:H2B-YFP (CALS7:H2B-YFP) marker visualizing nuclei of early protophloem cells prior to enucleation [24] was absent in **smxl4;smxl5** roots (Figures 3F and 3G). Taking these observations together, we concluded that early processes during SE formation are impaired in **smxl4;smxl5** RAMs. To demonstrate a context-independent role of **SMXL4/5** in phloem formation, we investigated phloem differentiation capacity of **smxl5** and **smxl4;smxl5** cotyledons employing VISUAL [23, 25]. Before trans-differentiation, phloem appearance was comparable between wild-type and **smxl5** and **smxl4;smxl5**

![Figure 2. smxl4;smxl5 Roots Lose RAM Activity over Time and Protophloem Differentiation Is Delayed](image)
mutants (Figures 3H–3J). After inducing trans-differentiation, phloem abundance was lower in both smxl5 and smxl4;smxl5 mutants when compared to wild-type (Figures 3K–3M) and the increase of phloem-associated gene activity was less pronounced (Figure 3N). Supporting a specific role in phloem formation, no robust difference in xylem formation (Figures S2A–S2F) or in the induction of xylem-related genes was found.

Because we suspected that SMXL3 activity masked a role of SMXL4/5 in phloem strand formation under non-induced conditions, we analyzed the non-treated progeny of a viable smxl3;smxl4/+;smxl5 plant. Indeed, 36% of smxl3;smxl4/+;smxl5 plants and 82% of smxl3;smxl4;smxl5 plants were devoid of phloem-related staining (Figures 3O–3T). Collectively, these findings indicated that SMXL3/4/5 genes are early key regulators of phloem formation contributing in a dose-dependent manner.

**Phloem Transport Capacities to the RAM Are Reduced in smxl4;smxl5 Plants**

To assess the impact of the observed defects on phloem-based transport to the RAM, we investigated phloem-dependent movement in wild-type and smxl4;smxl5 roots. Grafting of wild-type

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**Figure 3. Phloem Formation Is Impaired in smxl4;smxl5 and Absent in smxl3;smxl4;smxl5 Mutants**

(A–E) Characteristic longitudinal planes depicted from 3D volumetric SBEM datasets of differentiating protophloem strands from two individual roots. The dashed lines marked B–E indicate the locations of the cross-sections shown in (B)–(E). Developing protophloem strands are marked by yellow arrows and xylem poles by asterisks. Scale bars represent 10 μm in (B) and (D) and 1 μm in (C) and (E).

(F and G) Activity of the CALS7:H2B-YFP reporter. Scale bars represent 50 μm.

(H–J) Callose deposition in cotyledons visualized by aniline staining. Scale bars represent 100 μm.

(K–M) Aniline staining after VISUAL treatment [23] for 6 days. Scale bars represent 100 μm.

(N) After treatment (K–M), cotyledons of three independent experiments were used to determine transcript levels of genes. One-way ANOVA (CI 95%) with post hoc Bonferroni for datasets with homogeneous variances (MYB46, XCP1, NEN4, NAC086) or with post hoc Tamhane-T2 for datasets with inhomogeneous variances (IRX3, SEOR1, APL) was performed. Phloem-related genes are marked in blue, xylem-related genes in black.

(O–T) Cotyledons from 10-day-old plants shown under bright field (O–Q) or with aniline-based callose visualization (R–T). Scale bars represent 200 μm. Error bars represent ± SD. See also Figures S2 and S3.
shoots onto smxl4;smxl5 mutant roots did not improve root growth (Figures S2G and S2H). Interestingly, leaves of all grafts supported by smxl4;smxl5 roots were smaller and accumulated more sucrose while all leaves supported by wild-type roots had no defect (Figures S2I and S2J). Likewise, sugar exudation from smxl5 and smxl4;smxl5 leaves was not impaired but was according to the overall sugar levels found in our direct measurements (Figures S2J and S2K). Together, these observations showed that phenotypic alterations in shoots were a secondary effect of SMXL4/5 deficiency in roots.

Indeed, when wild-type shoots containing the SUCROSE TRANSPORTER 2:GREEN FLUORESCENT PROTEIN (SUC2:GFP) transgene [26] were grafted onto wild-type or smxl4;smxl5 roots, weaker GFP intensities were observed in tips of the longest smxl4;smxl5 root compared to wild-type root tips (Figures S2L and S2M). Of note, at the stage of analysis, the primary root of smxl4;smxl5 plants was shorter than the lateral roots and did not display any GFP fluorescence at the tip (Figures S2N and S2O). These findings indicated reduced phloem-dependent transport to the smxl4;smxl5 RAM.

SMXL4 and SMXL5 Do Not Mediate SL/KAR Signaling
To see whether SMXL4/5, like other SMXL family members [8, 10, 13, 14], act as strigolactone (SL) or KAR signaling mediators, we analyzed root growth and protophloem formation in max2 single and smxl4;smxl5:max2 double mutants. Interestingly, smxl4;smxl5:max2 mutants were indistinguishable from smxl4;smxl5 mutants in terms of root length and RAM size (Figures S3A–S3G). In particular, tangential cell divisions of SE precursor cells were as delayed in smxl4;smxl5:max2 as in smxl4;smxl5 double mutants (Figures S3H–S3L). Furthermore, max2 mutants initiated those divisions just like wild-type plants (Figures S3I and S3J). Likewise, formation of protophloem-derived SEs was similarly impaired in both smxl4;smxl5 and smxl4;smxl5:max2 plants (Figures S3M–S3P). Those findings argued for a MAX2-independent role of SMXL4 and SMXL5 in promoting phloem differentiation and RAM activity and against a role as mediators of MAX2-dependent SL/KAR signaling.

SMAX1 Can Functionally Replace SMXL5, but Only SMAX1 Stability Is SL/KAR Dependent
To see whether the lack of genetic interaction between SMXL4/5 and the SL/KAR signaling pathway is reflected by differences in SL/KAR-dependent protein degradation, we compared stabilities of SMXL3-YFP, SMXL4-YFP, SMXL5-YFP, and SMAX1-YFP fusion proteins expressed stably in plants. To exclude that different sites of protein accumulation affect SL/KAR responsiveness, we first expressed SMXL5-YFP and SMAX1-YFP fusion proteins under the control of the SMXL5 promoter in smxl4;smxl5 mutants. As a result, not only the SMXL5:SMAX1-YFP but also the SMXL5:SMAX1-YFP transgene suppressed the growth defect usually observed in smxl4;smxl5 roots (Figures 4A and 4B). This demonstrated that both proteins were active and, importantly, that SMAX1 was able to replace SMXL5 when present in the same cells. Interestingly, when grown with rac-GR24 triggering both SL and KAR signaling [12], the compensatory effect of the SMXL5:SMAX1-YFP transgene on smxl4;smxl5 root length was lost, while the SMXL5:SMXL5-YFP transgene was still able to fully restore growth of smxl4;smxl5 roots (Figures 4A and 4B).

To determine whether the rac-GR24-dependent difference between SMAX1-YFP and SMXL5-YFP was reflected by different protein stabilities, we investigated YFP intensities in root tips after short-term rac-GR24 treatments. Overlapping with sites of SMXL5 promoter activity (Figure 1), SMXL5-YFP and SMAX1-YFP proteins were localized to the nuclei of developing phloem cells and SMAX1-YFP and SMXL5-YFP levels were comparable when incubated without rac-GR24 (Figures 4C and 4E). As expected, the SMAX1-YFP signal started fading around 8 min after the onset of rac-GR24 treatments and was completely gone after 12 min (Figure 4D). In contrast, the SMXL5-YFP signal remained unaffected, even when treatment was maintained for 1 hr (Figures 4F and 4G). Similar to SMXL5-YFP, levels of SMXL3-YFP and SMXL4-YFP were unaffected by rac-GR24 when expressed under the control of their own promoters (Figure S4). Thus, stabilities of SMAX1 but not of SMXL subclade 2 members are rac-GR24 dependent.

DISCUSSION
In this study, we provide evidence that the Arabidopsis SMXL3, SMXL4, and SMXL5 genes promote phloem development. In addition, we provide evidence that SMXL3, SMXL4, and SMXL5 activities do not depend on SL/KAR signaling components acting on other SMXL family members. Collectively, we demonstrate a role of SMXL3/4/5 proteins as SL/KAR-independent developmental triggers of long-distance transport capacities.

Several functionally tightly interconnected genes have been identified to act in protophloem differentiation in the RAM [27]. Interestingly, none of the described phloem-defective mutants shows all the defects observed in smxl4;smxl5 plants. For example, similar to smxl4;smxl5, brevis radix (brx) mutants show a delay or absence of the second tangential cell division of the SE precursor cell lineage [28]. Similar defects are found in OCTOPUS (OPS)-deficient plants [29]. In both ops and brx mutants, protophloem differentiation is affected, which is reflected by the presence of undifferentiated “gap” cells alternating with differentiated SEs [18, 19]. This phenotype is common among several protophloem-defective mutants for which cotyledon vascular pattern 2 (cvp2);cvp2-like 1 (cv1) double mutants are another example [19]. Along the same lines, ALTERED PHLOEM DEVELOPMENT (APL)-defective mutants show defects in phloem differentiation but normal protophloem development including cell wall thickening, which is absent in smxl4;smxl5 [21, 30]. In contrast, smxl4;smxl5 plants display a general block of SE formation, suggesting that the SMXL4/5 gene functions are more central.

Interestingly, over-accumulation of CLAVATA3/ENDOSPERM SURROUNDING REGION 45 (CLE45) peptides triggers a similar block of SE differentiation as observed in smxl4;smxl5 mutants [18]. The putative receptor of CLE45 is the leucine-rich repeat receptor-like kinase BARELY ANY MERISTEM 3 (BAM3) [18]. However, we found the SMXL3/4/5 promoters to be active already in SE-pro cambium stem cells whereas CLE45 and BAM3 expression appears first in SE precursor cells [27]. This may argue for an earlier role of SMXL3/4/5 in phloem formation.

Our genetic analyses and analyses of SMXL3/4/5 protein stability suggest that the activities of SMXL4 and SMXL5 proteins do not depend on known components of the SL/KAR signaling
The observation that the sequence motif identified to be important for MAX2/D14-dependent ubiquitination \cite{6, 7} is not conserved in members of sub-clade 2 \cite{14, 31} is in line with this idea. In fact, reduction of SMXL4/5 activity did not result in the suppression of enhanced branching observed in max2 mutants, again arguing for an SL-independent role \cite{14}. Moreover, smxl4;smxl5;max2 mutants were indistinguishable from smxl4;smxl5 mutants, which argues against a MAX2-dependent contribution of other SMXL proteins to phloem development. However, this interpretation is based on the assumption that other SMXL proteins are under the control of SL/KAR signaling in early root development and, more specifically, in developing phloem cells. Because SL/KAR signaling naturally depends on endogenous SL/KAR levels which could be low in those cells at this stage, we cannot exclude that SL/KAR signaling regulates phloem formation at other times or places. In fact, phloem tissues proved to be perceptive to SL/KAR signaling but SMAX1-YFP levels were as high as SMXL5-YFP levels without rac-GR24 treatments.

In summary, beyond being essential mediators of SL/KAR signaling and plant growth plasticity \cite{32}, SMXL proteins are central regulators of phloem formation and general plant growth. The three SMXL sub-clades not only hold different regulatory roles, but also have fundamental differences in protein function. Independence of SMXL3, SMXL4, and SMXL5 from SL/KAR signaling may be crucial for a robust formation of long-distance transport capacities and, consequently, plant vitality.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.03.014.

**AUTHOR CONTRIBUTIONS**


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