

SMXL3 controls multiple aspects of Arabidopsis development via EAR motif-dependent and -independent functions

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SUMMARY

SMAX1-LIKE (SMXL) proteins, previously linked to strigolactone and karrikin signalling, play diverse and partially redundant roles in plant development. The divergent SMXL4 superclade—comprising SMXL3, SMXL4 and SMXL5—is not subject to strigolactone- or karrikin-dependent proteolysis. Although these proteins have been associated with phloem differentiation and primary root growth in *Arabidopsis thaliana*, their broader functions remain underexplored. In this present work, we used double mutants and a series of complementing lines to investigate the *in vivo* functions of SMXL3 and its prominent motifs. Loss of both SMXL3 and SMXL5 resulted in spontaneous adventitious root formation on the hypocotyl, a phenotype suppressed in etiolated seedlings and modulated by carotenoid-derived signals. In contrast, anchor root formation, maintenance of primary root tip dominance and vertical root growth orientation were redundantly controlled by all SMXL4 superclade members and required an intact EAR repression motif in SMXL3. Beyond the root system, SMXL3, SMXL4 and SMXL5 collectively influenced vegetative growth, while the combined activity of SMXL3 and SMXL4 limited higher-order inflorescence branching and promoted embryo development. Together, our findings reveal that SMXL4 superclade proteins act in a combinatorial manner to control developmental timing and root architecture through both EAR motif-dependent and -independent mechanisms, with root-associated phenotypes likely reflecting altered auxin redistribution or local auxin accumulation. Overall, SMXL proteins emerge as key components controlling plant development in a context-dependent manner.

Keywords: auxin, root architecture, SMAX-LIKE, strigolactones, EAR motif.

INTRODUCTION

SUPPRESSOR OF MAX2 1-LIKE (SMXL) proteins are multifunctional repressors that function either as non-transcriptional regulators through protein–protein interactions (Chang et al., 2024) or as transcriptional co-repressors that regulate gene expression, in part through their conserved ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motifs (Jiang et al., 2013; Wang et al., 2015; Zhou et al., 2013). EAR motifs recruit the transcriptional co-repressors TOPLESS (TPL) and TOPLESS-RELATED (TPR), which function broadly across plant signalling pathways by repressing transcription through chromatin- and transcriptional machinery-mediated repression (Plant et al., 2021). In some cases, SMXL proteins might function as transcription factors (Pan et al., 2024; Wang, Wang, et al., 2020). In Angiosperms,

members of the SMXL family are classified into two major superclades, SMAX1 and SMXL4 (Walker et al., 2019). In *Arabidopsis*, this family comprises eight genes. Within the SMAX1 superclade, the SMAX1 clade is represented by SMAX1 and SMXL2 in *Arabidopsis* and is primarily targeted by the karrikin signalling pathway through MORE AXILLARY GROWTH2 (MAX2) and KARRIKIN INSENSITIVE2 (KAI2) (Li et al., 2022; Wang, Xu, et al., 2020). This pathway plays crucial roles in controlling seed germination, seedling photomorphogenesis, primary root growth, root hair density, drought tolerance and the establishment of arbuscular mycorrhiza (Chang et al., 2024; Choi et al., 2020; Feng et al., 2023; Stanga et al., 2013; Villacéja-Aguilar et al., 2019; Zheng et al., 2020). By contrast, the members of the SMXL7/8 clade, which includes SMXL6, SMXL7 and SMXL8 in *Arabidopsis*, are targeted by DWARF14 (D14) and MAX2

in response to strigolactone signalling (Jiang et al., 2013; Zhou et al., 2013). This pathway governs diverse developmental and physiological processes, including seedling morphogenesis, shoot branching, leaf development and senescence, lateral root formation, stem elongation, secondary growth and responses to drought stress (Jiang et al., 2013; Liang et al., 2016; Soundappan et al., 2015; Wang et al., 2015; Zhou et al., 2013). Notably, SMAX1 and SMXL2 can also be targeted by the D14–MAX2 complex, indicating partial functional overlap between the karrikin- and strigolactone-mediated pathways (Li et al., 2022; Wang, Xu, et al., 2020).

The less-characterized SMXL4 superclade comprises the SMXL3/9 and SMXL4 clades, which are sister lineages that diverged independently from an ancestral SMAX1-like gene. The SMXL3/9 clade includes SMXL3 and SMXL9, the latter likely originating from a dicot-specific duplication event and therefore absent from monocots and, notably, the Brassicaceae (Walker et al., 2019). The SMXL4 clade itself consists of SMXL4 and SMXL5 in *Arabidopsis* (Walker et al., 2019). Unlike the SMAX1 superclade, SMXL4 superclade members are not targeted by strigolactone or karrikin signalling-mediated protein degradation (Wallner et al., 2017), consistent with the absence of the degron motif required for interaction with D14 and MAX2 (Jiang et al., 2013; Li et al., 2024; Moturu et al., 2018; Walker et al., 2019; Zhou et al., 2013). SMXL4 and SMXL5 expression was initially shown to be controlled by DCL2-dependent transcriptional silencing, and knockdown/knockout of these genes led to anthocyanin overaccumulation and phloem defects (Wu et al., 2017). SMXL4 and SMXL5 translation is regulated by the JULGI/5'UTR G-quadruplex module (Cho et al., 2018). A seminal study identified the primary functions of SMXL3, SMXL4 and SMXL5 as being crucial for phloem formation and the promotion of primary root growth (Wallner et al., 2017). Intriguingly, the *SMXL5_{PRO}:SMAX1* promoter swap construct rescues the primary root phenotype of *smxl4,5* mutants, suggesting that SMXL proteins might have interchangeable roles (Wallner et al., 2017). The primary root growth of *smxl4,5* is highly sensitive to strigolactone, and these double mutants exhibit reduced primary shoot branching (Li et al., 2024). SMXL5 was found to interact with D14 and SMXL7, indicating not only cross-clade interactions between co-repressors and their canonical signalling complexes but also associations among SMXL proteins from different superclades (Li et al., 2024). Consequently, SMXL5 could modulate strigolactone signalling by inhibiting SMXL7 degradation, partly through its conserved EAR motif (Li et al., 2024).

Remarkably, single mutants of these SMXL genes rarely display prominent phenotypes, or such observations have yet to be reported. *smxl5* and *smxl4,5* mutants lack secondary phloem formation, while *smxl4* and *smxl4,5*

mutants exhibit excessive stem cambial proliferation (Wallner et al., 2020). SMXL4 also positively influences flowering time, rosette size, seed set (Yang et al., 2020), and responses to salt and drought stress in the *Arabidopsis* C24 ecotype (Yang et al., 2015).

SMXL3 is the least studied member of the SMXL4 superclade. *smxl3* single mutants do not display any prominent phenotypes, and only double mutants with *smxl4* and *smxl5* have been reported to exhibit reduced primary root growth, suggesting functional redundancy. Given the broad vascular expression of SMXLs (Soundappan et al., 2015; Wallner et al., 2017; Wu et al., 2017), we hypothesized that the genetic interactions among members of the SMXL4 superclade might extend beyond the regulation of phloem formation and primary root growth, potentially manifesting as novel or enhanced phenotypes in double mutants. Furthermore, we proposed that the EAR motif of SMXL3 could modulate these composite phenotypes. To test these hypotheses, we examined a broad spectrum of root system architecture (RSA) phenotypes and investigated the role of the SMXL3 EAR motif in developmental control.

In this study, we report that double mutants exhibit an unexpectedly diverse range of phenotypes, indicating that members of the SMXL3 and SMXL4 clades collectively, and often in pairwise combinations, control multiple developmental processes, including adventitious root and anchor root formation, root trajectory and, additionally, shoot and embryo development. Moreover, we demonstrate that the conserved C-terminal EAR motif of SMXL3 is required for the modulation of RSA but is dispensable for the control of aerial development.

RESULTS

Functional analysis of conserved SMXL3 regions and motifs

The domain structure of SMXL4 superclade members differs slightly from that of the SMAX1 superclade (Moturu et al., 2018; Walker et al., 2019). The functions of the SMXL4 and SMXL5 EAR motifs have thus far been only partially characterized (Li et al., 2024; Pan et al., 2024). Therefore, we first examined the contribution of major structural features of the SMXL3 protein to its function in *Arabidopsis*, using primary root growth as a phenotypic readout. Though the conserved motif and domain structure of SMXL3 resemble other SMXL proteins, it, along with SMXL4 and SMXL5, lacks the RGKT degron motif (Moturu et al., 2018; Walker et al., 2019), which is required for the MAX2-mediated degradation of SMAX1 and SMXL7/8 clade members (Jiang et al., 2013; Khosla et al., 2020; Soundappan et al., 2015; Wang et al., 2015; Wang, Xu, et al., 2020; Zhou et al., 2013). Additionally, SMXL3 and SMXL9 completely lack a part of a second

C-terminal ATPase domain (D2b) (Walker et al., 2019), which is thought to be involved in SMAX1 protein–protein interactions (Khosla et al., 2020). To identify highly conserved regions and motifs, we compiled 148 angiosperm SMXL3 sequences together with the closely related SMXL9 (Table S1) and used AlphaFold3 (Abramson et al., 2024) to predict the tertiary structure of SMXL3, thereby identifying candidate regions likely to adopt distinct folded conformations. Sequence conservation among angiosperm SMXL3 orthologues aligns well with AlphaFold-predicted structural features and with the major domains of Arabidopsis SMAX1 as defined by Khosla et al. (2020) (Figure 1a,b). Although AlphaFold predictions for SMXL3 should be considered as structural guides rather than definitive domain boundaries, the predicted folded regions largely coincide with conserved sequence blocks, interspersed with disordered regions, consistent with previous observations (Khosla et al., 2020; Temmerman et al., 2022). The consensus EAR motif at the C-terminus of SMXL3 is LDLNx in dicots and (L/F/I)DLNx in monocots, while SMXL9 proteins conserve LDLNx (Figure 1c). Although the DLN box in SMXL3 is highly conserved, the fifth-position leucine (L) is less conserved, particularly in monocots. Moreover, the EAR motifs in both SMXL3 and SMXL9 lack the nearby proline (P), suggesting a divergent, intermediate-type EAR motif between the LxLxL and DLNxP types (Kagale & Rozwadowski, 2011; Yang et al., 2018).

To assess the contributions of the major domains and the EAR motif of SMXL3 (Uniprot ID: Q9SVD0) *in vivo*, we generated a series of lines expressing YFP-tagged deletion constructs in *smx/3,5* background (Figure 1a; Table S2). These included an N-terminal Clp-N domain deletion (Δ Clp-N), in which two short, highly conserved segments predicted to be located on the surface of the domain were removed between positions 70–162 and 391–507. The N-terminal domain has been suggested to mediate protein–protein interactions with transcription factors and to be involved in specifying SMXL functions (Chang et al., 2025a). Additionally, we generated five-amino-acid deletions in the predicted degenerate ATPase regions: the highly conserved GDLNW motif in the D1 region, which resembles a DLN box (Δ D1), and the intermediate-type EAR motif (LDLNL) located in the C-terminal D2 region (Δ EAR). Previous studies suggest that the D1 region, together with the intermediate M region, may contribute to conferring specificity to SMXL interactions with the strigolactone and karrikin receptors D14 or KAI2 (Khosla et al., 2020). To reduce potential steric effects of the deletions, we created alanine substitution mutants for Δ D1 (Δ D1Ala) and Δ EAR (Δ EARAla), replacing each motif with the mutated sequence 'ADANA' in both cases.

We found that the *SMXL3* ^{Δ Clp-N} construct failed to rescue the short primary root phenotype associated with the *smx/3,5* mutations (Figure 1d,e), and no YFP signal

appeared in root vasculature nuclei (Figure 1d). While this result is consistent with a role of the N-terminal region in protein stability or accumulation, alternative explanations, including transcriptional or translational effects, misfolding or altered protein turnover, could also be considered. Likewise, Δ D1 and Δ D1Ala did not complement *smx/3,5*, though YFP signal was detected in vasculature, suggesting protein stability and that the DLN box-like motif residing on the D1 domain contributes to SMXL3 function (Figure 1d,e).

Unlike Δ Clp-N, lines carrying Δ EAR or Δ EARAla constructs displayed strong YFP signals and partially rescued the stunted primary root growth phenotype (Figure 1d,e). Given that SMXL family members can physically interact (Khosla et al., 2020; Li et al., 2024) and that SMXL4 and SMXL5 form heterodimers (Pan et al., 2024), we hypothesized that the function of the SMXL3 EAR motif may depend on the genetic context, particularly on the presence or absence of functional SMXL4 or SMXL5. To test this, we introduced the Δ EAR variant into the *smx/3,4* background. Intriguingly, the primary root length phenotype associated with Δ EAR in *smx/3,5* background were observed in *smx/3,4*, but the primary root phenotype was intermediate between the fully complemented lines and those complemented in *smx/3,5* (Figure 1e), suggesting that the function of SMXL3 is partially modulated by the EAR motif through SMXL4 and SMXL5.

As the structure–function analysis revealed a context-dependent role of the SMXL3 EAR motif in the control of primary root growth, we asked whether the loss of this motif could contribute to other RSA-related phenotypes potentially arising in double mutants. To this end, we examined these combinations for prominent phenotypic alterations that could serve as readouts for SMXL3 EAR motif function. Unexpectedly, the double mutants displayed a broad spectrum of phenotypes affecting the RSA. Given the pleiotropic and complex nature of the phenotypes associated with the loss of SMXLs and the EAR motif of SMXL3, we describe these findings in detail in the following sections.

The combination of SMXL3 and SMXL5 exerts opposite effects on adventitious root formation in etiolated and non-etiolated hypocotyls

Upon manipulating young seedlings, we noticed that extensive adventitious root formation occurs spontaneously in intact, non-etiolated hypocotyls of light-grown *smx/3,5* mutants (Figure 2a–c and Figures S1). Adventitious roots form on aerial parts of plants either naturally or in response to environmental cues. In Arabidopsis, their formation is normally highly restricted and triggered by factors such as wounding, loss of the root apical meristem, high humidity or low light, with auxin acting as a central regulator of initiation (Boerjan et al., 1995; Li, 2021; Negi

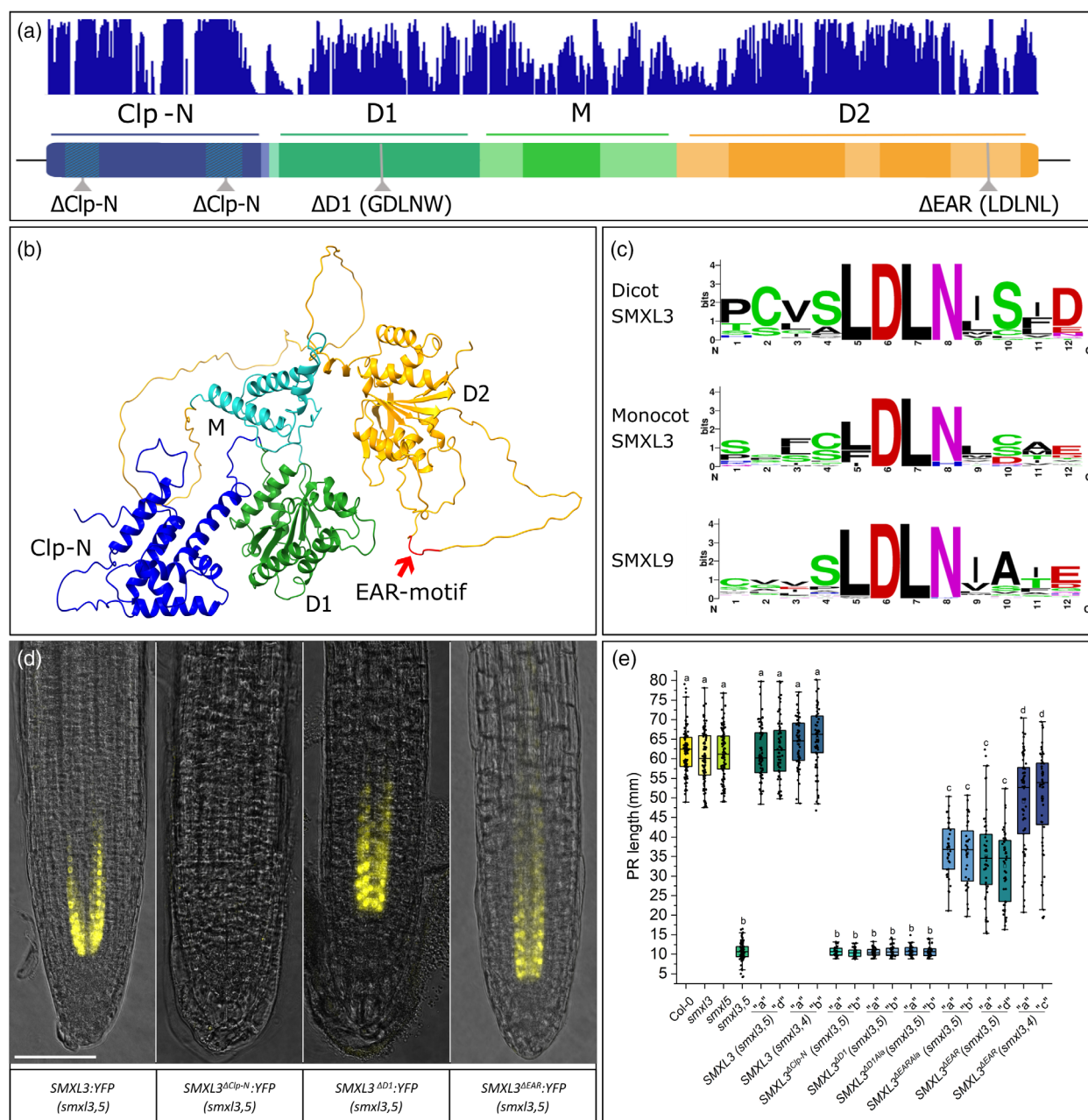


Figure 1. Δ Clp-N and Δ D1 constructs fail to restore the Col-0 phenotype in *smx13,5* background.

(a) Conservation of Angiosperm SMXL3 protein sequences and the predicted domain architecture of SMXL3 are shown. Sequence alignment of multiple Angiosperm SMXL3 protein sequences has been performed with the built-in ClustalW platform of Ugene software. Proposed major domains are annotated according to Khosla et al. (2020). Deleted Clp-N (Δ Clp-N) regions, the EAR-like (Δ D1) and C-terminal EAR (Δ EAR) motifs are highlighted in grey.

(b) Three-dimensional structure of Arabidopsis SMXL3 protein predicted by AlphaFold3. Major domains are indicated as per Figure 1a.

(c) The WebLogo (<https://weblogo.berkeley.edu/>) illustrates the conserved C-terminal EAR motif in dicot and monocot SMXL3, as well as Angiosperm SMXL9.

(d) Confocal images of primary root (PR) tips from 7-day-old seedlings of representative transgenic lines: *SMXL3_{PRO}:SMXL3::YFP*, *SMXL3_{PRO}:SMXL3 ^{Δ Clp-N}::YFP*, *SMXL3_{PRO}:SMXL3 ^{Δ D1}::YFP* and *SMXL3_{PRO}:SMXL3 ^{Δ EAR}::YFP* in the *smx13,5* background. No YFP signal was detected in mutants complemented with Δ Clp-N constructs. Scale bar = 50 μ m.

(e) Δ Clp-N, Δ D1 and Δ D1A1a constructs failed to complement the short PR phenotype of *smx13,5*. PR lengths of 14-day-old *SMXL3_{PRO}:SMXL3*, *SMXL3_{PRO}:SMXL3 ^{Δ Clp-N}*, *SMXL3_{PRO}:SMXL3 ^{Δ D1}*, *SMXL3_{PRO}:SMXL3 ^{Δ D1A1a}* and *SMXL3_{PRO}:SMXL3 ^{Δ EAR}* plants grown on vertical 1/2 MS agar plates with sucrose. Box-and-whisker plots with identical letters are not significantly different ($n = 60$; ANOVA, $P < 0.01$; Games-Howell multiple comparisons test). Independent transgenic lines are labelled with letters in double quotation marks across all figures. Additional data regarding the complementation of other *smx13,5*-associated phenotypes are presented in Figure S3A-D.

et al., 2010; Sukumar et al., 2013). Adventitious roots are formed in the aerial parts of plants either naturally or in response to changes in environmental conditions. Under normal conditions, adventitious root formation in *Arabidopsis* is highly restricted and initiated by wounding, loss of root apical meristem, humidity and low light (Li, 2021). Auxin has a central role in adventitious root initiation (Bellini et al., 2014; Boerjan et al., 1995; Negi et al., 2010; Sukumar et al., 2013). Intriguingly, the spontaneous adventitious roots formed in *smx1/3,5* were predominantly located in the basal parts of the hypocotyl (Figure 2b and Figure S1). In parallel, no adventitious root formation has been observed in other lines, suggesting that SMXL3 and SMXL5 act specifically and non-redundantly in this context to suppress adventitious root formation. Strigolactones have been shown to counteract auxin activity during adventitious root induction (Rasmussen et al., 2012) possibly via regulating local auxin levels, suggesting that adventitious root initiation could arise from altered auxin–strigolactone balance. Members of the SMXL4 superclade have been suggested to be associated with strigolactone and karrikin signalling pathways (Végh et al., 2017; Machin et al., 2020). A direct role of SMXL5 in strigolactone-related signalling has also been demonstrated recently (Li et al., 2024). Based on these findings, we hypothesized that the spontaneous adventitious root formation in *smx1/3,5* mutants may involve changes in auxin accumulation or modified interactions with strigolactone signalling. To test this, we supplemented the culture medium with either the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA)—a well-established inhibitor of directional auxin transport (Abas et al., 2021; Teale et al., 2021) that effectively suppresses adventitious root formation (Sukumar et al., 2013)—or the strigolactone-mimicking compound *rac*GR24 (Rasmussen et al., 2012). Both NPA and *rac*GR24 could effectively inhibit adventitious root emergence in *smx1/3,5* mutants, indicating that adventitious root formation in these mutants depends on auxin transport and is suppressed by strigolactone signalling (Figure 2c). Because *rac*GR24 can activate both strigolactone signalling and the paralogous karrikin signalling pathway (Scaffidi et al., 2014), we further tested the effects of the natural strigolactone stereoisomer (+)-5-deoxystrigol ((+)-5DS) and karrikin2 (KAR2) (Figure 2c). These treatments specifically induce signalling either through the D14 strigolactone receptor ((+)-5DS) or the KAI2 karrikin receptor (KAR2). We observed that (+)-5DS suppressed adventitious root formation, whereas KAR2 had no detectable effect, suggesting that adventitious root induction in *smx1/3,5* plants is primarily controlled by strigolactone signalling under these conditions. Since anchor and lateral root formation is blocked by norflurazon (NF; Jia et al., 2019), a potent carotenoid biosynthesis inhibitor (Moise et al., 2014), we considered that NF might block

adventitious root formation in *smx1/3,5* mutants. Surprisingly, NF inhibited adventitious root formation in *smx1/3,5* mutants, suggesting that adventitious root emergence in these mutants also depends, at least in part, on a carotenoid-derived compound (Figure 2c).

Next, we asked whether the potential for adventitious root initiation differs among mutant combinations. A widely used approach to investigate adventitious root formation capacity is to expose etiolated seedlings to light, which typically triggers extensive root development along the hypocotyl (Sorin et al., 2005). To apply this method, seedlings were grown in the dark on vertical plates for 4 days and then transferred to light for at least 7 days to monitor adventitious root formation along the previously etiolated hypocotyl. Given the spontaneous adventitious root formation observed in light-grown *smx1/3,5* seedlings, we expected that *smx1/3,5* mutants would display an enhanced adventitious rooting response following etiolation and re-illumination. Surprisingly, no adventitious roots were detected in *smx1/3,5* even 7 days after re-illumination, whereas adventitious roots were initiated along the entire de-etiolated hypocotyl in all other genotypes tested (Figure 2d,f and Figure S1). Extending the light exposure by 3 additional days resulted in a few adventitious roots in the de-etiolated hypocotyl segment, while roots were abundant in the non-etiolated apical segment (Figure 2e,g and Figure S1). Together, these observations reveal a paradoxical, context-dependent role for the SMXL3/SMXL5 combination: while SMXL3 and SMXL5 suppress spontaneous adventitious root formation in light-grown, non-etiolated hypocotyls, they are required for efficient adventitious root initiation in response to etiolation and subsequent light exposure. Recently, the novel diapocarotenoid compound anchorene was identified as an endogenous promoter of adventitious root formation in *Arabidopsis* (Ke et al., 2024). Given that anchorene acts through the inhibition of indole-3-acetic acid (IAA) inactivation (Ke et al., 2024) and NF inhibited adventitious root formation in light-grown *smx1/3,5* seedlings, we tested whether anchorene application could restore adventitious root formation in etiolated *smx1/3,5* seedlings. Intriguingly, anchorene partially restored *smx1/3,5* phenotype toward that of Col-0, suggesting that anchorene can at least partially compensate for the inhibition of adventitious root initiation in etiolated hypocotyls of *smx1/3,5* seedlings (Figure 2h).

Finally, we investigated whether the EAR motif of SMXL3 is required for controlling adventitious root formation. In the *smx1/3,5* background, seedlings of light-grown *SMXL3^{ΔEAR}* lines formed no adventitious roots (Figure 2a,c and Figure S1) suggesting that the EAR motif of SMXL3 does not contribute to the suppression of adventitious root formation.

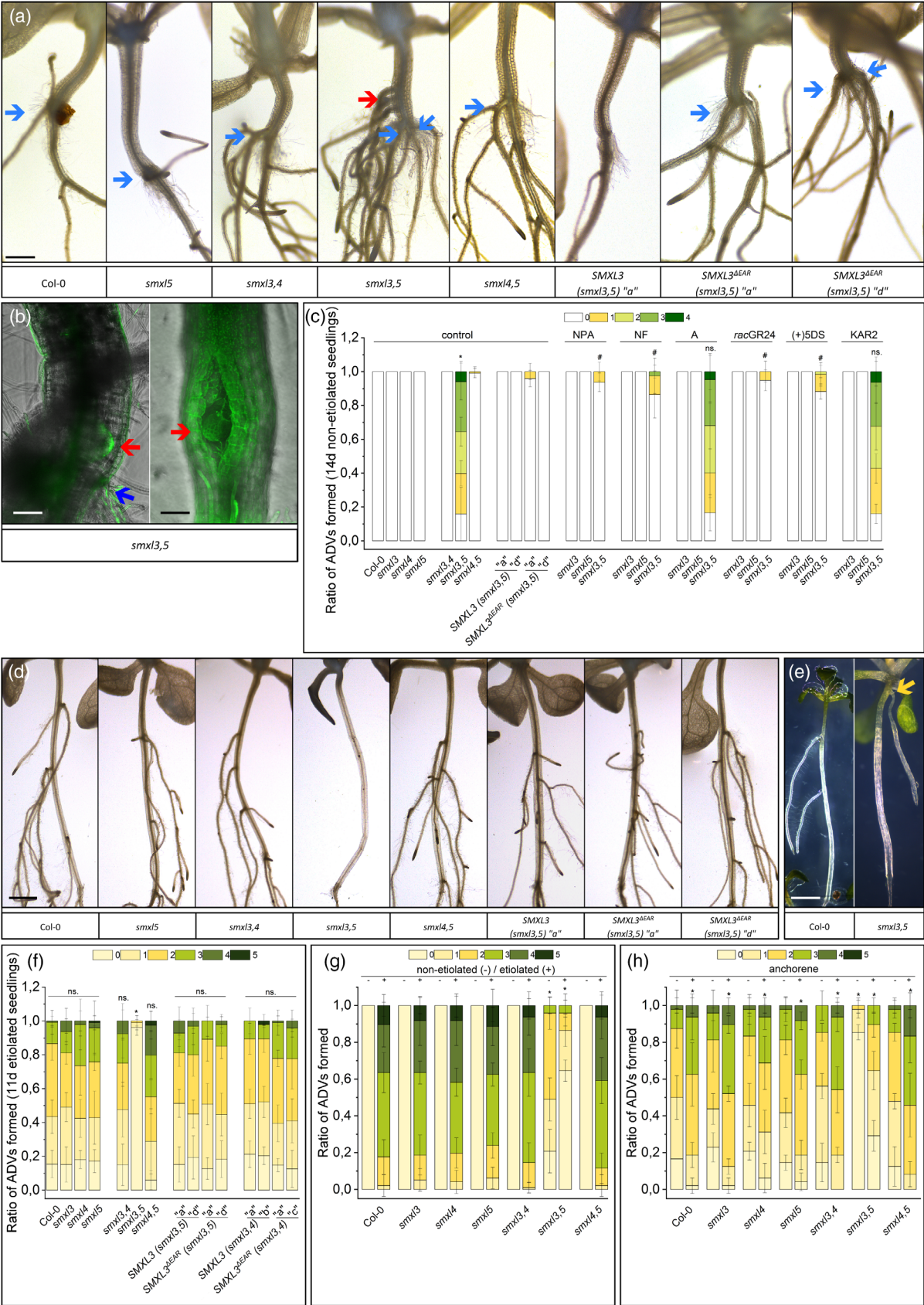


Figure 2. The SMXL3/SMXL5 combination exerts opposite effects on adventitious root formation in etiolated and non-etiolated hypocotyls.

- (a) Adventitious roots (ADVs) are formed spontaneously in the hypocotyls of *smx1/3,5* seedlings. Morphology of non-etiolated hypocotyls and the collet region of 14-day-old seedlings grown vertically on ½ MS agar plates with sucrose. Arrows indicate anchor roots (ARs; blue) and ADVs (red). Plants were mounted in ethanol. Bar = 0.5 cm.
- (b) Morphology of ADV primordia (left panel) and newly emerged ADVs (right panel) in 11-day-old *smx1/3,5* seedlings grown vertically on ½ MS agar plates with sucrose. For auxin buildup visualisation, plantlets were incubated in 5 µM FluorA I. for 30 min, and fluorescence has been detected by CLSM. Arrows indicate the collet region (blue) and ADV (red). Bar = 100 µm.
- (c) Ratio of ADVs formed in non-etiolated hypocotyls after treatments with NPA, NF, anchorene, *rac*-GR24, (+)5DS, and KAR2. The proportion of plants forming 0 to 4 ADVs is indicated by different colours. Plants were grown vertically on ½ MS agar plates with sucrose. Asterisks (*) indicate significant differences from Col-0 control; hash symbols (#) indicate differences from *smx1/3,5* control plants (4–9 biological replicates with 12 plants per replicate; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).
- (d) Adventitious root (ADV) formation is inhibited in the etiolated hypocotyls of *smx1/3,5* seedlings. Morphology of etiolated hypocotyls of 14-day-old seedlings grown on vertical ½ MS agar plates with sucrose. The proportion of plants forming 0 to 5 ADVs is indicated by different colours. Plants were kept in the dark for 4 days, then transferred to light for 10 days. Bar = 0.5 cm.
- (e) Morphology of etiolated hypocotyls of 18-day-old Col-0 and *smx1/3,5* seedlings grown on vertical ½ MS agar plates with sucrose. The proportion of plants forming 0 to 5 ADVs is indicated by different colours. Plants were kept in the dark for 4 days, then transferred to light for 14 days. Yellow arrow indicates ADV formed in the non-etiolated segment of *smx1/3,5* hypocotyl. Bar = 0.5 cm.
- (f) Ratio of ADVs in etiolated hypocotyls 7 days after light exposure. Plants were grown on vertical ½ MS agar plates with sucrose. The proportion of plants forming 0 to 5 ADVs is indicated by different colours. Asterisks denote significant differences from Col-0 (mean ± SD; 4–9 biological replicates with 12 plants each; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).
- (g) Ratio of ADVs in etiolated and non-etiolated hypocotyls 14 days after light exposure. The proportion of plants forming 0 to 5 ADVs is indicated by different colours. Asterisks denote significant differences from Col-0 (mean ± SD; 4–9 biological replicates with 12 plants each; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).
- (h) Ratio of ADVs in etiolated hypocotyls 10 days after light exposure and anchorene treatment. After 4 days in dark, seedlings were transferred to vertical ½ MS agar plates with sucrose, supplemented with 20 µM anchorene. The proportion of plants forming 0 to 5 ADVs is indicated by different colours. Asterisks denote significant differences from Col-0 control (mean ± SD; 4–9 biological replicates with 12 plants each; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).

The EAR motif of SMXL3 is essential for the efficient suppression of anchor root formation

Anchor roots represent a specialized root type arising at the hypocotyl-root junction, or collet (Jia et al., 2019; Scheres et al., 1994), initiated from pericycle cells positioned on opposite sides of the collet and oriented along the cotyledons (Jia et al., 2019). Environmental cues, such as soil texture and nutrient availability, along with auxin and the recently identified anchorene, regulate anchor root formation (Jia et al., 2019). We found that the initiation of single and double anchor roots is strongly promoted in all mutant combinations, suggesting that SMXL4 superclade members redundantly suppress anchor root emergence (Figures 2a and 3a). Over time, anchor roots were rapidly initiated in *smx1/3,4* and *smx1/4,5*, while in *smx1/3,5*, anchor root formation occurred substantially later (Figure 3c). Both NPA and NF were shown to effectively block anchor root formation (Jia et al., 2019). Testing the responses to these chemicals revealed that all three mutant combinations were sensitive to NPA, however, partially insensitive to NF treatments (Figure 3a), indicating that anchor root formation in SMXL4 superclade mutants depends on auxin transport and is at least partially independent of carotenoid biosynthesis.

We also tested whether *rac*GR24, which acts in parallel with SMXL3 and SMXL5-dependent adventitious root suppression, affects anchor root formation. The strigolactone mimic had no effect on anchor root formation in any genotypes, indicating that strigolactones are unlikely to play a role in anchor root suppression (Figure 3b). Additionally, we examined whether anchorene treatment would

further increase anchor root formation capacity in the mutant combinations (Jia et al., 2019). The anchorene treatment uniformly elevated the number of plants with one or two anchor roots across all genotypes (Figure 3b).

Finally, we evaluated the number of anchor roots in 14-day-old *SMXL3^{ΔEAR}* lines. The loss of the EAR motif did not fully restore the wild-type anchor root suppression phenotype in either mutant background, indicating that the EAR motif is required for effective suppression of anchor root formation (Figures 2a and 3a). Similar to the double mutants, the EAR motif mutants were sensitive to NPA but partially insensitive to NF treatments, suggesting that the EAR motif contributes to anchor root suppression both by affecting auxin redistribution and via a mechanism partially dependent on carotenoid synthesis (Figure 3a).

SMXL3 EAR motif is required for maintaining primary root tip dominance

Double mutants were previously described as short-root mutants implying that the members of the SMXL4 superclade act redundantly (Wallner et al., 2017). The authors reported that the primary roots of *smx1/4,5* plants appeared shorter than the LR. By 14 days after germination, however, double mutants exhibit a more complex and divergent RSA (Figure 4a,b and Figure S2A). All mutant combinations displayed prominent anchor or lateral root formation, with root lengths comparable to or even exceeding the primary root (Figure 4a–c). In particular, *smx1/4,5* showed relatively long anchor or lateral roots, whereas *smx1/3,5* exhibited the shortest root lengths

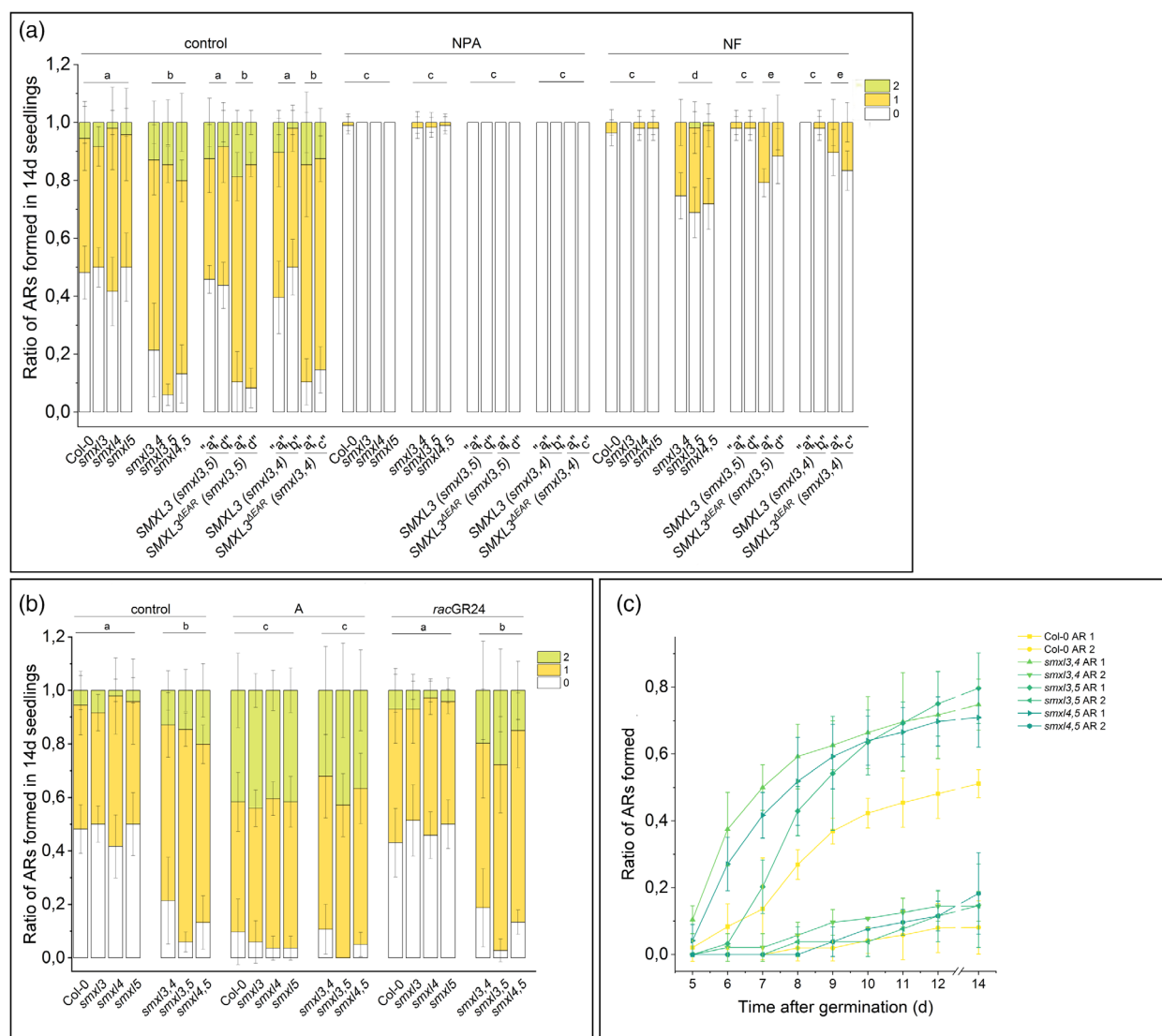


Figure 3. SMXL4 superclade members redundantly suppress anchor root formation.

(a) The EAR motif of SMXL3 is essential for suppression of anchor root (AR) formation. Ratio of ARs in the collet region following 1 μ M NPA and 1 μ M NF treatment is shown. Seedlings were grown on vertical $\frac{1}{2}$ MS agar plates with sucrose. The proportion of plants forming 0 to 2 ARs is indicated by different colours. Bars with the same letter are not significantly different (mean $\pm$ SD; 4–8 biological replicates with 12 plants each; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).

(b) Ratio of ARs in the collet region after 20 μ M anchorene and 1 μ M *rac*GR24 treatments. Seedlings were grown on vertical $\frac{1}{2}$ MS agar plates with sucrose. The proportion of plants forming 0 to 2 ARs is indicated by different colours. Bars with the same letter are not significantly different (mean $\pm$ SD; 4–8 biological replicates with 12 plants each; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).

(c) Ratio of ARs formed over time in Col-0 and double mutant combinations grown on vertical ½ MS agar plates with sucrose. Single (labelled as AR 1) or double (AR 2) ARs were counted separately.

(Figure 4a,b), suggesting that primary root tip dominance is compromised in these mutants. In *SMXL3^{1EAR}* lines, primary root length was only partially restored to Col-0 or single mutant levels, and the dominance of the primary root over lateral and anchor roots was not fully recovered, indicating that SMXL3 plays a critical role in maintaining primary root growth and dominance over lateral and anchor roots via its EAR motif (Figure 4a–c). Additionally, we observed that in the double mutants, the first lateral root

was located very close to the collet, within a 0.5 mm range, whereas in Col-0 and single mutants, the first lateral root typically emerged much further away (Figure 4d). To facilitate the distinction between anchor roots and proximal lateral roots, we stained collet hairs with toluidine blue following Janková Drdová et al. (2019), and scored proximal lateral roots when they appeared within 0.5 mm of the collet (a practical cut-off threshold). Notably, these phenotypes were not fully complemented in the *SMXL3*^{1^{EAR}}

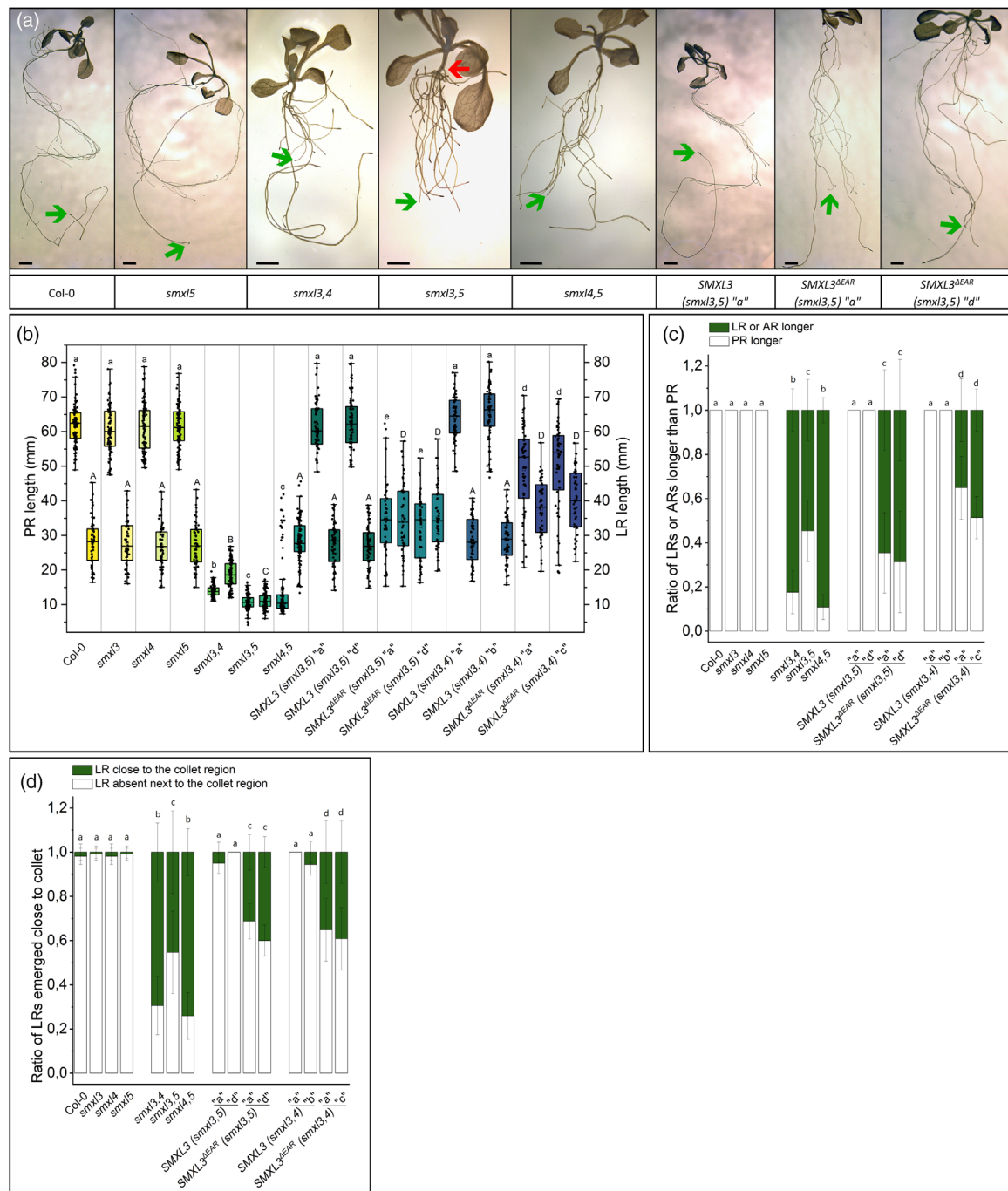


Figure 4. SMXL3 contribute to the maintenance of primary root tip dominance through its EAR motif.

(a) Lateral root (LR) or anchor root (AR) growth often predominates over primary root (PR) growth in double mutants and *SMXL3^{ΔEAR}* lines. Root morphology of 14-day-old plants grown on vertical ½ MS agar plates with sucrose. Plants were mounted in ethanol. Arrows indicate PRs (green) and adventitious roots (ADVs; red). Bar = 2 mm. Note that *smxl3,4*, *smxl3,5* and *smxl4,5* plants were photographed at twice the magnification.

(b) PR and LR or AR lengths of 14-day-old plants grown on vertical ½ MS agar plates with sucrose. The length of the longest LR or AR was measured. For each plant line, the two data groups correspond to measurements plotted on the left and right y-axes, respectively. Box-and-whisker plots with the same letter are not significantly different. Lowercase and uppercase letters indicate statistical groups for PR and LR lengths, respectively ($n = 60-96$; ANOVA, $P < 0.01$; Games-Howell multiple comparisons test).

(c) Ratio of seedlings in which LR or AR length exceeds PR length. Ratio has been calculated using the data presented in Figure 4b. Plants were grown for 14 days on vertical ½ MS agar plates with sucrose. Bars with the same letter are not significantly different (mean $\pm$ SD; 6 biological replicates with 12 plants each; Kruskal-Wallis non-parametric test, $P < 0.01$; Dunn-Bonferroni test).

(d) Ratio of LRs emerging near the collet region (within 0.5 mm) in 14-day-old seedlings grown on vertical ½ MS agar plates with sucrose. Bars with the same letter are not significantly different (mean $\pm$ SD; 6 biological replicates with 12 plants each; Kruskal-Wallis non-parametric test, $P < 0.01$; Dunn-Bonferroni test).

lines, further underscoring the importance of the EAR motif in maintaining normal RSA (Figure 4d).

The EAR motif of SMXL3 is required for maintaining normal root growth trajectory

Gravity is the primary determinant of root growth direction; however, this trajectory is further modulated by environmental factors such as water and nutrient availability, salt stress and physical impedance (Lee et al., 2020; Sun et al., 2008; Xie et al., 2021). Deviations from straight, downward root growth typically manifest as wavy or curling root trajectory patterns (Grabov et al., 2005; Roy & Basham, 2014) and are associated with impaired auxin signalling and distribution (Sun et al., 2008; Zhang & Friml, 2020; Xie et al., 2021). When examining root length parameters, we found that the primary roots of *smx1/3,5* mutants, as well as the primary roots, anchor and lateral roots of *smx1/4,5* mutants, exhibited a wavy root phenotype (Figure 5a,b). The vertical growth ratio (VGR), defined as the ratio of total root length (L_t) to vertical root length (L_v), confirmed that the growth trajectory is compromised in these mutant combinations. Surprisingly, *SMXL3^{1EAR}* lines exhibited an even more severe gravitropism deficiency, particularly in the *smx1/3,5* background (Figure 5a,b). Similar to anchor root, the phenotype in *smx1/3,4* background was less pronounced, further suggesting that the function of the *SMXL3^{1EAR}* motif is context-dependent (Figure 5a,b). Because the VGR of *SMXL3^{1EAR}* lines exceeds the VGR observed in *smx1/3,5*, we considered whether this might reflect a gain-of-function mutation. To test this hypothesis, we crossed *SMXL3^{1EAR}* (*smx1/3,5*) lines with *smx1/5* mutants. All heterozygous plants displayed wild-type VGR, demonstrating that *SMXL3^{1EAR}* represents a recessive loss-of-function mutation (Figure 5c). Root waving is partially linked to auxin redistribution defects (Lee et al., 2020; Sun et al., 2008; Xie et al., 2021; Zhang & Friml, 2020). Therefore, we applied 1 μ M NPA to the roots (Rashotte et al., 2000), hypothesizing that the waving would be more pronounced in *SMXL3^{1EAR}* lines if auxin availability was compromised. As expected, *SMXL3^{1EAR}* lines displayed a severe waving and even a curling phenotype, consistent with this prediction (Figure 5b and Figure S2B,C). To test

whether auxin distribution is altered in *SMXL3^{1EAR}* lines, we applied the 7-nitro-2,1,3-benzoxadiazole fluorophore (NBD)-labelled derivative of 2,4-D, FluorA II., to assess tissue-specific auxin distribution in 5- to 7-day-old seedlings (Pařízková et al., 2021). In *SMXL3* (*smx1/3,5*) and *SMXL3^{1EAR}* (*smx1/3,5*) plants with normal gravitropism, fluorescence patterns were comparable to Col-0 and single mutants (Figure 5d). In contrast, spontaneously agravitropic primary roots of *smx1/3,5* and the EAR mutants showed strong FluorA II. accumulation, indicating that auxin redistribution in primary roots is compromised (Figure 5d). Interestingly, the application of NF partially rescued the straight root trajectory in *SMXL3^{1EAR}* lines (Figure 5b and Figure S2D) suggesting that this phenotype is influenced, at least in part, by a carotenoid-derived signal involved in controlling root growth trajectory.

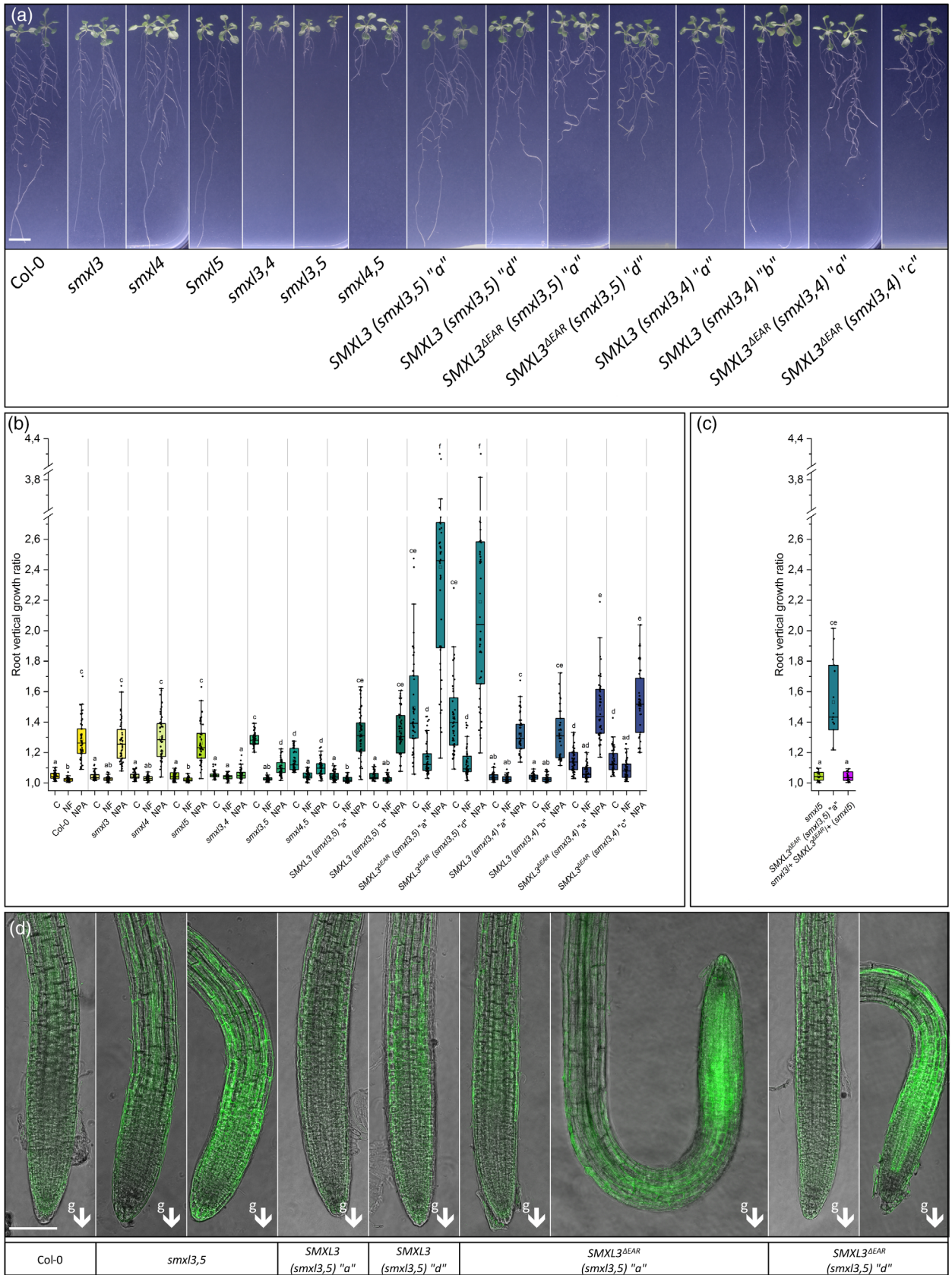
SMXL4 superclade members contribute to the control of stature and branching architecture

The comprehensive analysis of root-associated phenotypes revealed that the belowground functions of SMXL4 superclade members are largely redundant and highly dependent on a functional EAR motif in SMXL3. In contrast, adventitious root suppression requires functional SMXL3 and SMXL5 but is independent of a functional SMXL3 EAR motif. We then asked whether the loss of function of any SMXL4 superclade proteins might lead to aerial phenotypes, whether these potential phenotypes act in pairwise combinations or redundantly, and whether they depend on a functional SMXL3 EAR motif.

Since the *SMXL4* mutant *Athspr* in ecotype C24 displays reduced rosette area and moderate delay in flowering (Yang et al., 2020), we first assessed these traits in long-day-grown single and double mutants and *SMXL3^{1EAR}* lines in Col-0 background. Rosette area was measured on day 22, when ~50% of the Col-0 and single mutant plants had started flowering, and on day 28, when the double mutants had already started flowering (Figure 6a,b and Figure S3A,B). All double mutants displayed smaller rosettes at day 22, especially *smx1/3,5*, but later these lines partially caught up with Col-0 and single mutant lines.

Figure 5. The EAR motif of SMXL3 is required to establish normal root growth trajectory.

(a) Primary roots (PRs) of *SMXL3^{PRO}:SMXL3^{1EAR}* lines exhibit a wavy growth trajectory. Root morphology of 14-day-old intact seedlings is presented. Seedlings were grown on vertical 1/2 MS agar plates with sucrose. Bar = 1 cm.
 (b) Vertical growth ratio (VGR) of the PR in 14-day-old plants grown on 1/2 MS agar plates supplemented with either 1 μ M NF or 1 μ M NPA. VGR was calculated as the ratio of total root length (L_t) to vertical root length (L_v). Box-and-whisker plots with the same letter are not significantly different ($n = 64$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test).
 (c) VGR of the PR in 14-day-old *smx1/5*, *SMXL3^{PRO}:SMXL3^{1EAR}* (*smx1/3,5*) and *smx1/3/+ SMXL3^{PRO}:SMXL3^{1EAR}/+ smx1/5* heterozygous plants grown on 1/2 MS agar plates. VGR was calculated as the ratio of total root length (L_t) to vertical root length (L_v). Box-and-whisker plots with the same letter are not significantly different ($n = 10$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test).
 (d) FluorA II. fluorescence intensity in PR tips of 7-day-old seedlings. Plants were grown vertically on 1/2 MS agar plates. Samples were incubated in 1/2 MS solution supplemented with 5 μ M FluorA II. for 15 minutes. For *smx1/3,5* and *SMXL3^{PRO}:SMXL3^{1EAR}* lines, PRs exhibiting gravitropic (left panel) and agravitropic growth (right panel) are shown. ‘g’ and the arrow indicate the direction of the gravity vector. 5–10 seedlings (in three biological replicates) were examined and the frequency of those displaying the presented FluorA II. accumulation pattern was ~80%. Bar = 100 μ m.



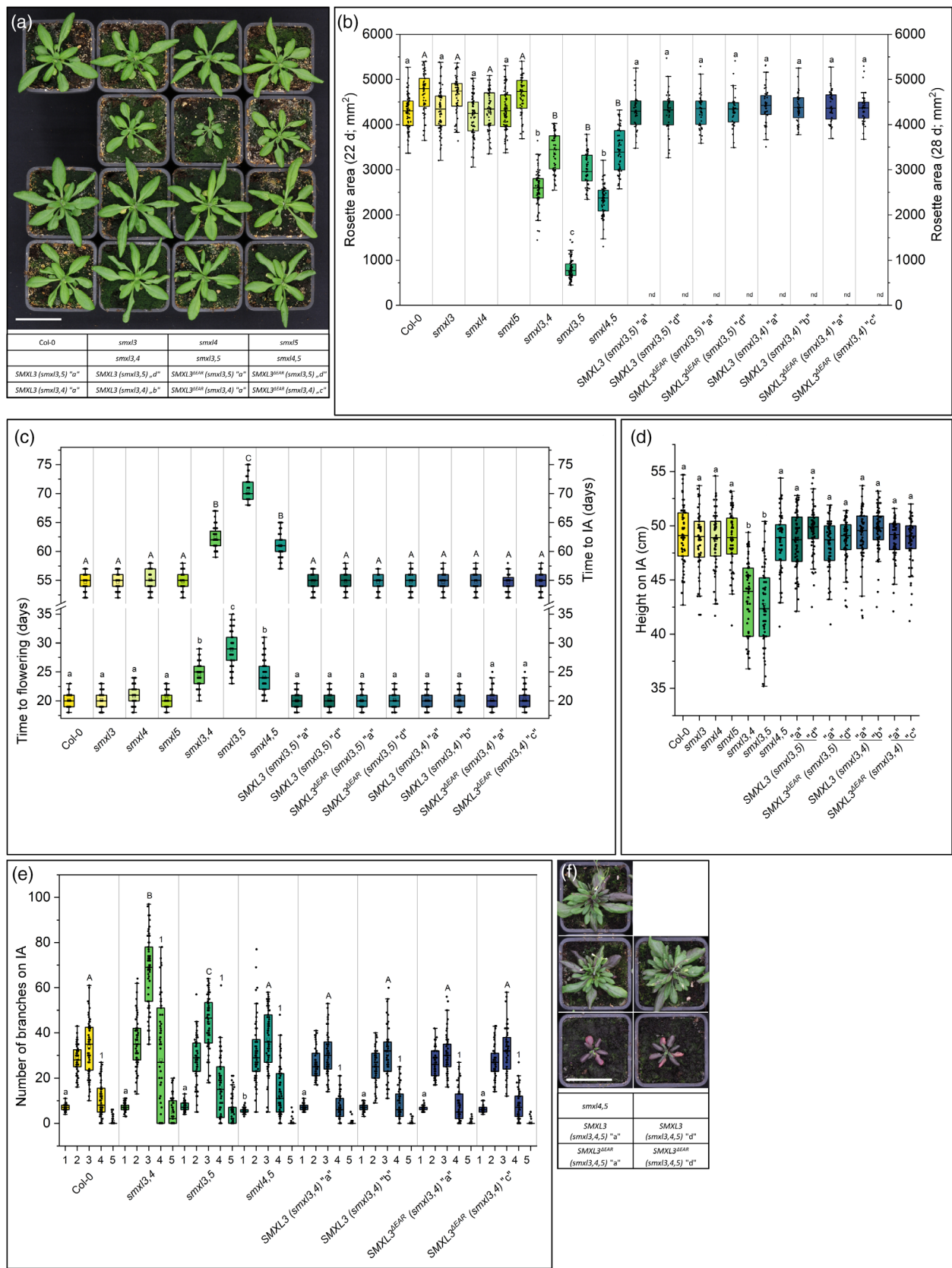


Figure 6. Members of the SMXL4 superclade control vegetative growth, floral transition and branching.

- (a) Rosette morphology of 22-day-old plants grown under long-day conditions (16 h light/8 h dark). Scale bar = 5 cm.
- (b) Rosette area is reduced and growth is delayed in double mutant lines. Rosette area has been assessed in 22 and 28-day-old plants grown under long-day conditions. For each plant line, the two data groups correspond to measurements plotted on the left and right y-axes, respectively. Lowercase and uppercase letters distinguish statistical groups for rosette area for 22 and 28-day-old plants, respectively ($n = 60$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test).
- (c) Time to flowering and time to reach inflorescence arrest (IA). Plants were grown under long-day conditions. For each plant line, the two data groups correspond to measurements plotted on the left and right y-axes, respectively. Data were collected from 80 plants per genotype. Lowercase and uppercase letters indicate statistically distinct groups for time to flowering and time to IA, respectively ($n = 80$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test).
- (d) *smxl3,4* and *smxl3,5* plants exhibit reduced height at IA. Height was measured on the day the terminal flower opened. Plants were grown under long-day conditions. Data were collected from 60 plants per genotype. Box-and-whisker plots with identical letters are not significantly different ($n = 60$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test).
- (e) *smxl3,4* plants exhibit an increased number of higher-order branches. Plants were grown under long-day conditions. Data were collected from 64 plants per genotype on the day of IA. Primary branch number includes the primary shoot. The x-axis indicates branching order (1st to 5th) in each genotype. Box-and-whisker plots with identical letters are not significantly different. Lowercase, uppercase and numerical labels indicate statistically distinct groups for primary, tertiary and quaternary branches, respectively ($n = 64$; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).
- (f) The rosette phenotype of 30-day-old *smxl4,5*, *SMXL3^{PRO}:SMXL3* (*smxl3,4,5*) and *SMXL3^{PRO}:SMXL3^{1EAR}* (*smxl3,4,5*) plants grown under long-day conditions (16 h light/8 h dark). Scale bar = 5 cm.

SMXL4 and SMXL5 repress transcription of *CHALCONE SYNTHASE (CHS)* in a dual-LUC reporter assay in an EAR motif-dependent manner (Pan et al., 2024). Since CHS is a key enzyme in anthocyanin biosynthesis, and *smxl4,5* rosettes accumulate high levels of anthocyanins (Li et al., 2024; Wu et al., 2017), we examined whether other mutant combinations might display a similar phenotype. Anthocyanin accumulation was most pronounced in 15-day-old seedlings, particularly in *smxl3,5* rosettes (Figure S5A). To assess later developmental stages, we collected the 6th and 7th leaves from mature rosettes. At this stage, *smxl4,5* plants showed the strongest anthocyanin accumulation, whereas *SMXL3^{1EAR}* lines were indistinguishable from the respective single mutants (Figure S5B, C). These findings indicate that, unlike SMXL4 and SMXL5, the EAR motif of SMXL3 is likely not required for the control of anthocyanin accumulation.

Next, we examined flowering termination, height and branching structure at the stage of primary inflorescence arrest (Walker et al., 2023). All three double mutant combinations exhibited a significant delay in bolting time, with *smxl3,5* being particularly delayed in initiating flowering (Figure 6c and Figure S4A). Neither the rosette area nor the flowering time in single mutant lines differed significantly from Col-0, suggesting that a single *smxl4* mutation is not sufficient in the Col-0 background to induce these phenotypes. All three double mutant combinations showed a marked delay in reaching primary inflorescence arrest, with *smxl3,5* exhibiting the most substantial effect (Figure 6c and Figure S4B,C). *SMXL3^{1EAR}* lines again displayed the Col-0 phenotype. Interestingly, while *smxl3,4* and *smxl3,5* displayed reduced stature, *smxl4,5* plants were not different from Col-0 (Figure 6d). Additionally, we assessed the anthocyanin content of dry inflorescences. Again, all combinations accumulated anthocyanin, with *smxl4,5* being the most prominent in this regard (Figure S5B,C). Together, these findings demonstrate that SMXL4 superclade members redundantly control rosette

development, anthocyanin biosynthesis and the reproductive transition, and contribute to maintaining appropriate timing of developmental checkpoints in Arabidopsis. In contrast, the SMXL3 EAR motif does not appear to play a role in controlling these processes.

Having been putatively associated with strigolactones, we investigated whether SMXL4 superclade members contribute to the control of branching. To this end, we counted branch numbers at the stage of IA. Consistent with the recent observation (Li et al., 2024), *smxl4,5* plants displayed a slightly reduced number of axillary branches, while other lines and *SMXL3^{1EAR}* lines were indistinguishable from Col-0 and single mutants (Figure 6e, Figure S4D). Interestingly, *smxl3,4* plants have denser inflorescence, raising the possibility of a higher number of higher-order branches in this line. Surprisingly, *smxl3,4* plants exhibited a significantly higher number of tertiary branches compared to other lines (Figure 6e), suggesting that SMXL3 and SMXL4 together suppress higher-order branches and intriguingly do not affect primary rosette branching. Although cauline branching is highly correlated to flowering time (Fichtner et al., 2022), the strongest tertiary branching occurred in *smxl3,4* rather than in the more late-flowering *smxl3,5*, indicating a specific SMXL3–SMXL4 genetic effect. Furthermore, the phenotype was rescued by the *SMXL3^{1EAR}* construct in *smxl3,4*, indicating that the EAR motif is not essential for the suppression of third-order branches (Figure 6e and Figure S4D).

Finally, to assess whether the EAR motif is required for the essential functions of SMXL3, we crossed *SMXL3^{1EAR}* (*smxl3,5*) with *smxl3,4* to generate *SMXL3^{1EAR}* (*smxl3,4,5*) plants. *smxl3,4,5* plants are seedling-lethal (Wallner et al., 2017). Triple mutants harbouring the *SMXL3^{PRO}:SMXL3^{1EAR}* construct displayed severe developmental defects, including early rosette growth arrest, strong anthocyanin accumulation, stunted inflorescences and no seed production (Figure 6f). These results demonstrate that full SMXL3 function in the absence of SMXL4

and SMXL5 requires an intact EAR motif, underscoring its strict context-dependence.

SMXL3 and SMXL4 together contribute to silique and embryo development

Upon manipulation, we noticed that *smxl3,4* and *smxl3,5* plants displayed alterations in silique dimensions. Since *Athspr* has reportedly shorter siliques and reduced seed set (Yang et al., 2020), we tested whether the mutants and their combinations are compromised in silique, embryo and seed development. To assess silique traits, we uniformly harvested green siliques 13–14 days post-anthesis, when the embryos were in the mature green stage (Hu et al., 2018). We found that *smxl3,5* siliques were wider, while *smxl3,4* siliques were substantially shorter than those of Col-0 (Figure S6A,B). Next, we assessed whether these altered silique parameters were reflected in seed production. Interestingly, *smxl3,4* siliques exhibited a higher incidence of aborted and abnormal (smaller, slightly deformed or moderately shrunken) seeds at this stage of development (Figure 7a,b). The *SMXL3^{ΔEAR}* construct in *smxl3,4* background fully complemented the phenotype (Figure 7a). We speculated that the reduction in normal seeds might be due to defects in male/female reproduction or in embryo development. To test these scenarios, we first performed reciprocal crosses between *smxl3* or *smxl4* individuals and *smxl3,4* plants. All reciprocal fertilization events between the single and double mutants resulted in full restoration of the *smxl3* and/or *smxl4*-type seed set (Figure 7c), indicating that the reduced normal seed set is not a consequence of a fertilization defect, but rather associated with embryo development. Therefore, we assessed the expression of SMXL4 superclade members in embryos. We found that all three SMXLs displayed strong expression in the vasculature of torpedo-stage embryos (Figure 7d), in line with previous findings (Wallner, 2018). Next, we analysed embryo morphology using cleared whole-mount specimens. Embryos

in Col-0, single mutant, *smxl3,5* and *smxl4,5* were predominantly in the mature green stage, while *smxl3,4* seeds contained embryos at different developmental stages. Normal, wild-type seeds contained mature green stage embryos, whereas abnormal seeds from *smxl3,4* contained embryos at globular, heart, torpedo or bending torpedo stages (Figure 7e). Finally, we sowed after-ripened seeds (excluding aborted seeds) of *smxl3,4* and found that they germinated at a similar ratio to Col-0 seeds (Figure 7f). These findings collectively suggest that embryo development in smaller/shrunken seeds is not arrested but instead delayed. Thus, SMXL3 and SMXL4 together ensure normal seed and embryo development. Intriguingly, silique and seed parameters in single mutant lines were indistinguishable from Col-0, indicating that *smxl4* in Col-0 background under long-day conditions is apparently not sufficient to induce these phenotypes alone.

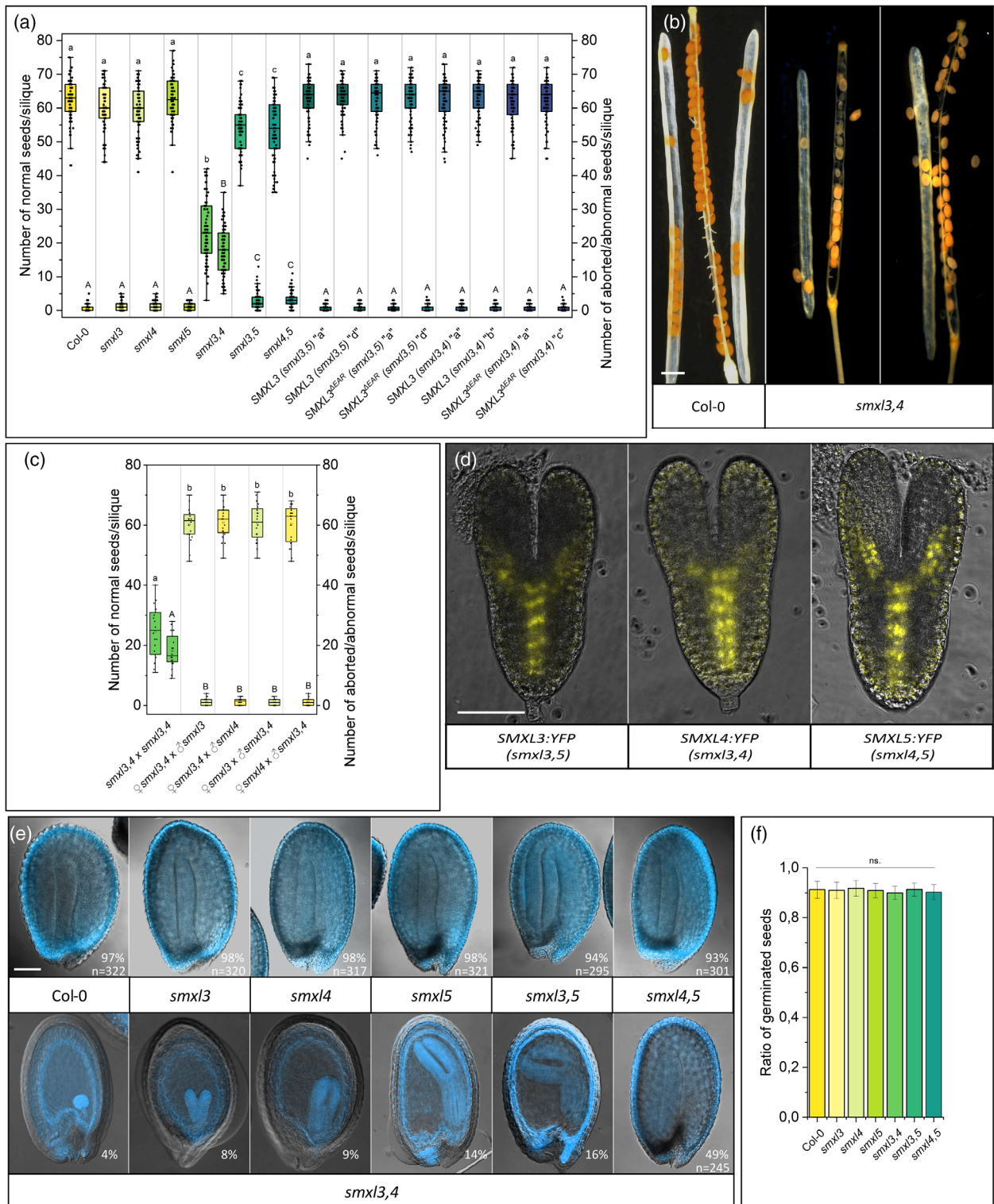
DISCUSSION

SMXL4 superclade members can act in pairwise combinations to control various developmental processes

We demonstrated that single mutants show no significant phenotypic changes, whereas combined mutations produce pleiotropic and a diverse array of seemingly unrelated phenotypes (Figure 8). This suggests that SMXL4 superclade members act beyond phloem-related functions with effects spanning multiple developmental processes. Consistent with earlier studies (Pan et al., 2024; Wallner et al., 2017), they display partial redundancy, as all mutant combinations exhibit delayed development and slower progression through key stages such as flowering and the transition to primary inflorescence arrest (Figure 8). Common traits include anthocyanin accumulation, reduced primary root length, excessive anchor root formation and impaired root dominance. However, the timing and severity of these phenotypes vary: *smxl3,5* mutants develop

Figure 7. The SMXL3-SMXL4 combination contributes to embryo development.

- (a) The number of normal seeds is reduced in *smxl3,4* green siliques. Seed counts were performed on green siliques collected at stage 13–14 days post-anthesis (DPA). Siliques were harvested and mounted in modified Hoyer's solution, and seeds were counted and assessed for morphology under a microscope. For each plant line, the two data groups (number of normal/abnormal seeds) correspond to measurements plotted on the left and right y-axes, respectively. Box-and-whisker plots with the same letter are not significantly different. Lowercase and uppercase letters differentiate statistical groups of normal and abnormal seeds (smaller, moderately shrunken, slightly deformed or aborted) ($n = 80$; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).
- (b) Morphology of Col-0 and *smxl3,4* seeds collected at stage 13–14 DPA. Siliques were mounted in modified Hoyer's solution.
- (c) Number of seeds in green siliques collected at stage 13–14 DPA of individuals from reciprocal crosses between *smxl3* or *smxl4* and *smxl3,4* plants. For each plant line, the two data groups correspond to measurements plotted on the left and right y-axes, respectively. Box-and-whisker plots with the same letter are not significantly different. Lowercase and uppercase letters indicate statistical groups of normal and abnormal seeds, respectively ($n = 20$; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).
- (d) SMXL3, SMXL4 and SMXL5 are expressed in torpedo-stage embryos. Confocal images of torpedo-stage embryos were obtained from lines harbouring *SMXL3_{PRO}::SMXL3::YFP*, *SMXL4_{PRO}::SMXL4::YFP* and *SMXL5_{PRO}::SMXL5::YFP* constructs. Bar = 50 μ m.
- (e) Seeds of *smxl3,4* contain embryos at various developmental stages. DIC images of embryos extracted from siliques at 13–14 DPA are presented. Siliques were mounted in modified Hoyer's solution, then, autofluorescence (violet) was detected using CLSM. The percentage of embryo types are shown at the bottom. 'n' indicates the total number of embryos examined for each genotype. Bar = 100 μ m.
- (f) Germination ratios of after-ripened seeds. Ten fully developed siliques were harvested (aborted seeds were removed), sterilized and sown on ½ MS agar plates. Germinated seeds were counted after one week. The experiment was repeated three times (mean $\pm$ SD; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test).



particularly slowly and *smxl4,5* accumulate high levels of anthocyanins. Although these traits are broadly shared among mutant combinations, certain phenotypes appear more sensitive to the loss of specific SMXLs. For example, loss of SMXL5 tended to be associated with stronger

effects on root trajectory and primary root length, SMXL4 with primary root dominance and SMXL3 with overall plant stature. These differences likely reflect variation in expression levels, tissue activity or interaction strength rather than strict functional specialization and will require

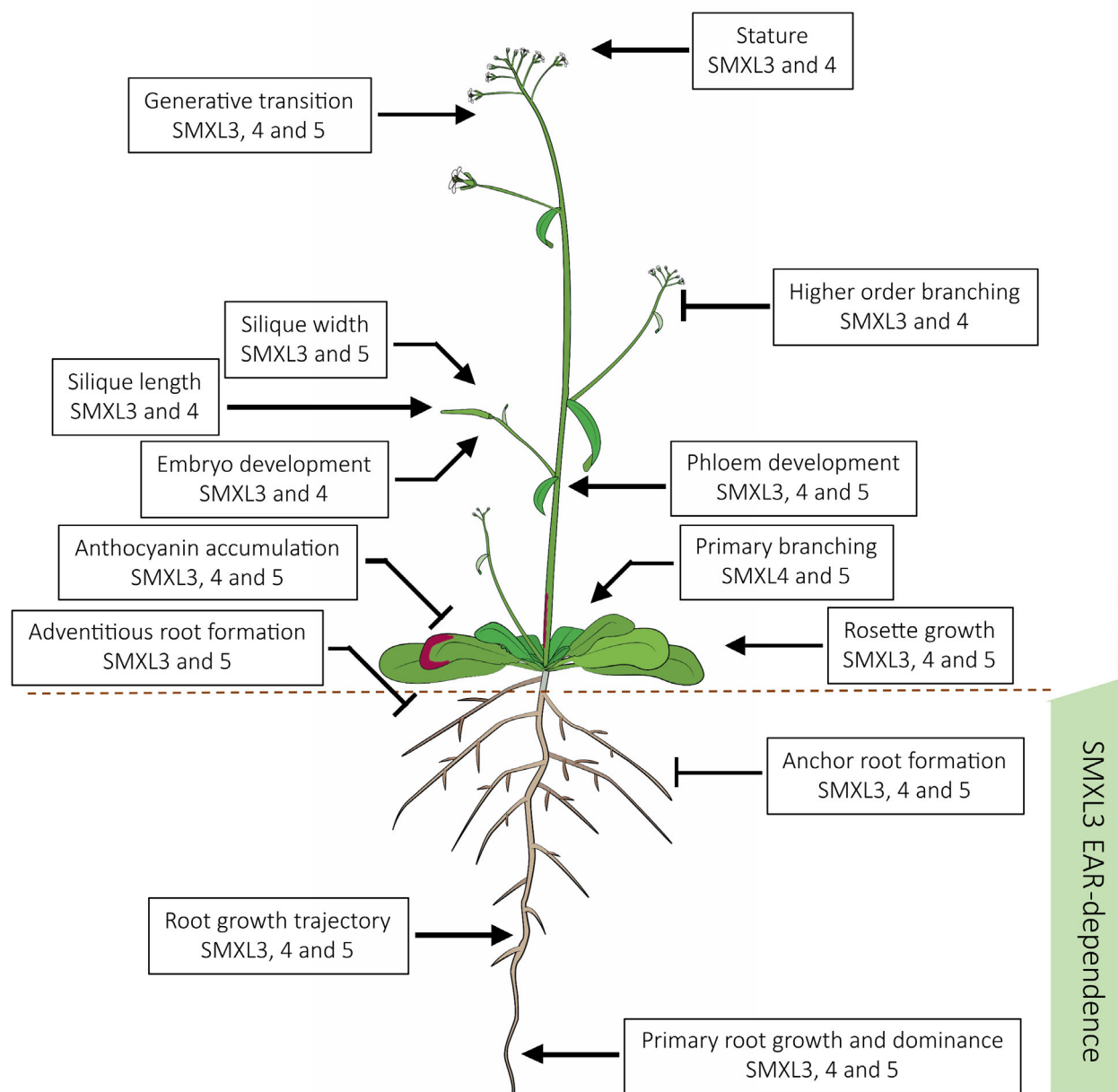


Figure 8. Scheme of the effects of SMXL4 superclade members on developmental processes in Arabidopsis under normal growth conditions. Members of the SMXL4 superclade act redundantly in the Arabidopsis Col-0 ecotype to contribute to multiple aspects of plant development. In many cases, SMXL3, SMXL4 and SMXL5 collectively contribute to the control of developmental processes. For example, proper phloem development (Wallner et al., 2017), rosette growth, flowering time, anthocyanin accumulation (Li et al., 2024; Wu et al., 2017), anchor root formation, primary root growth and dominance and root trajectory all require at least two functional members of the SMXL4 superclade. In other cases, specific SMXL combinations act synergistically to control distinct phenotypes. SMXL3 and SMXL4 are essential for normal embryo development, seed set, plant stature and modulation of higher-order branching. SMXL3 and SMXL5 primarily suppress adventitious root formation and influence silique width, whereas SMXL4 and SMXL5 act together to control primary branching (Li et al., 2024). Interestingly, SMXL3 appears to function in an EAR motif-dependent manner within the root system, but independently of the EAR motif in aerial tissues. Sketch of Arabidopsis plant was downloaded from FigShare (Frédéric Bouché [2018]: <https://figshare.com/ndownloader/files/13175255>). Blunt-ended arrows indicate negative effects, while pointed arrows indicate positive effects.

targeted future analyses to resolve. There are also striking cases where mutant combinations do not exhibit full redundancy. *smxl4,5* exhibit a reduced level of primary branches, consistent with the SMXL4 and SMXL5-mediated attenuation of strigolactone signalling (Li

et al., 2024). In contrast, *smxl3,4* mutants develop numerous higher-order branches (Figure 8). SMXL4 has been reported as a positive contributor to silique development in the C24 ecotype (Yang et al., 2020). In Col-0, *smxl4* resembles wild type, but *smxl3,4* mutants display smaller

siliques, reduced seed set and delayed embryo development (Figure 8), suggesting that loss of *SMXL4* alone is insufficient to cause reproductive defects, whereas additional loss of *SMXL3* is enough to trigger this phenotype. Notably, spontaneous adventitious root formation occurs exclusively in *smxl3,5* seedlings (Figure 8). Together, these results indicate that, beyond redundancy, *SMXL4* superclade members form pairwise combinations that might modulate each other's activity or assemble into complexes to fine-tune development.

How does collaboration among *SMXL4* superclade members manifest? As co-repressors, *SMXLs* engage in numerous physical associations with other proteins. *SMXL7* readily associates with *SMXL6* (Liang et al., 2016) and, intriguingly, with *SMXL5* and *SMA1* (Khosla et al., 2020; Li et al., 2024). Moreover, *SMA1*, *SMXL2* and *SMXL7* are capable of self-association, forming multimers (Khosla et al., 2020). Notably, the EAR motif appears to promote *SMXL7* multimerization, thereby enhancing its stabilization and association with *SMXL5*, ultimately resulting in attenuation of strigolactone signalling (Li et al., 2024). More recently, *SMXL4* and *SMXL5* were shown to associate, possibly as heterodimers (Pan et al., 2024). *SMA1* and *SMXL7* localize to nuclear speckles, where *SMXL7* primarily interacts with *D14* (Liang et al., 2016; Soundappan et al., 2015). It is intriguing to consider that the biomolecular condensates underlying these speckles may provide an additional platform for *SMXL* protein activity (Temmerman et al., 2022). Overall, *SMXL* proteins appear to assemble into higher-order multivalent structures that act as controlling or regulatory hubs (Chang et al., 2025b; Li et al., 2025).

An emerging view suggests that *SMXL* proteins function as *bona fide* transcription factors, adding a regulatory depth to their biological effects. *SMXL6*, *SMXL7* and *SMXL8* function as transcription factors, negatively regulating their own expression to modulate strigolactone signalling (Wang, Wang, et al., 2020). Similarly, *SMXL4* and *SMXL5* can directly bind to the promoter of *HEAT-SHOCK TRANSCRIPTION FACTOR A2 (HSFA2)* to regulate heat responses (Pan et al., 2024).

SMXL4 superclade members may also collaborate indirectly through parallel transcriptional regulons distinctly linked to *SMXL3*, *SMXL4* or *SMXL5*. While limited, available data suggest that these *SMXLs* regulate separate gene sets (Wallner et al., 2017; Wallner et al., 2020). Notably, *smxl4,5* mutants show a significant loss of phloem-specific open chromatin regions compared to *smxl5* alone, supporting cooperative gene regulation by *SMXL4* and *SMXL5* (Wallner et al., 2023).

A key question is whether the phenotypes in double mutants mainly reflect the activity of the intact, functional *SMXL* protein, with the absence of the other two proteins having a lesser impact. In this context, a cohort of *SMXL*

proteins, even across superclades, may collectively form a regulatory hub in which they could interfere with each other's activities (Chang et al., 2025b; Khosla et al., 2020; Li et al., 2024). This hub could involve a physical association of *SMXL* proteins, parallel transcriptional regulons or both. The loss of any two *SMXL4* superclade proteins might allow the remaining third protein to dominate, leading to the observed phenotypes.

EAR-dependent and EAR-independent functions of SMXL3

Loss of the EAR motif in *SMXLs* does not yield consistent phenotypes. For instance, in *SMXL7^{ΔEAR}* mutants, not all phenotypes associated with *SMXL7* loss are fully restored (Liang et al., 2016). *SMXL6* represses *BRANCHED1 (BRC1)* expression via EAR-dependent action (Wang, Wang, et al., 2020). Similarly, the *SMXL5^{ΔEAR}* construct partially rescues the short primary root and anthocyanin accumulation phenotypes in *smxl4,5* plants but fails to complement shoot branching and root *GR24^{5DS}* hypersensitivity, indicating that the EAR mutation is hypomorphic (Li et al., 2024). Recently, the repression of *HSF2A* transcription by *SMXL4/SMXL5* was shown to be EAR independent (Pan et al., 2024). In line with these findings, *SMXL3^{ΔEAR}* lines exhibit a spectrum of phenotypic complementation, ranging from partially dependent to fully EAR-independent traits (Figure 8). Notably, similar to *SMXL7* (Liang et al., 2016), *SMXL3^{ΔEAR}* variants still could influence all phenotypes to some degree, though these differences are minor and statistically insignificant under the conditions tested.

In aerial tissues, *SMXL3* functions largely independently of the EAR motif. We hypothesize that these embryo- and shoot-active, EAR-independent functions of *SMXL3* may involve non-transcriptional mechanisms, such as protein-protein interactions. Similar mechanisms have been described for *SMA1*, which directly binds phytochrome B (phyB) through its Clp-N domain to control hypocotyl thermomorphogenesis (Park et al., 2022). *SMA1* also interacts with *PIF4* and *PIF5* and suppresses their association with phyB (Chang et al., 2024), thereby regulating red-light responses. We speculate that in roots, *SMXL3* recruits repressors, such as *TPL* and *TPR* family proteins through its C-terminal EAR motif and the EAR-mediated transcriptional repressor activity predominates. The root-associated functions of the *SMXL3* EAR motif are influenced by genetic context, with phenotypic effects being more pronounced in the *smxl5* background than in *smxl4*. This suggests that EAR-dependent *SMXL3* function in roots is tuned by downstream processes modulated by *SMXL4* and/or *SMXL5*. Alternatively, the EAR motif may modulate cooperative interactions among superclade members. Consistent with this idea, the *SMXL5* EAR motif enhances the interaction between *SMXL5* and *SMXL7* *in vitro*, indicating a role for the EAR motif in protein-protein

interactions beyond those involving TPL and TPR family proteins (Li et al., 2024). However, similar to the weak interaction observed between SMXL5^{EAR} and SMXL7 (Li et al., 2024), SMXL3^{EAR} could still retain activity through EAR-independent interactions with SMXL4, SMXL5 or other interacting partners. Overall, SMXL3 function is largely EAR-independent in shoots but EAR-dependent in roots (Figure 8). Its EAR-dependent root function is therefore likely modulated by cooperative regulatory mechanisms among superclade members. Whether such cooperation involves the formation of SMXL-containing protein complexes, direct transcriptional activity of SMXL3, SMXL4 and SMXL5, or indirect regulation through parallel transcriptional regulons remains to be determined.

SMXL4 superclade members potentially modulate auxin redistribution in roots

In Arabidopsis, spontaneous adventitious root formation on the hypocotyl is rare and has been reported primarily in auxin-overproducing mutants such as *sur1*, *sur2* and *yucca1* (Boerjan et al., 1995; Zhao et al., 2001). Notably, *smx1/3,5* mutants similarly develop adventitious roots spontaneously in light-grown, non-etiolated hypocotyls and display enhanced sensitivity to the auxin transport inhibitor NPA. Together, these observations are consistent with a role for SMXL3 and SMXL5 in suppressing adventitious root initiation under normal light conditions, potentially by limiting auxin accumulation or auxin responsiveness in the hypocotyl. Paradoxically, despite this repressive role in light-grown seedlings, the combination of SMXL3 and SMXL5 is required for efficient adventitious root formation in etiolated hypocotyls following re-illumination. Recent work has identified the diapocarotenoid anchorene as an endogenous promoter of adventitious root formation in etiolated hypocotyls acting by increasing endogenous auxin levels through inhibition of IAA conjugation (Ke et al., 2024). In line with this, NF, which inhibits carotenoid biosynthesis, blocked adventitious root formation in light-grown *smx1/3,5* mutants, while exogenous anchorene partially restored adventitious rooting in etiolated *smx1/3,5* seedlings. These findings suggest that SMXL3 and SMXL5 are required to confer competence for adventitious root initiation in etiolated hypocotyls, and anchorene can partially bypass this requirement, likely via restoring free auxin levels. Thus, the *smx1/3,5* mutant exhibits opposite phenotypes depending on developmental and light context. This contrast indicates that SMXL3 and SMXL5 do not function as constitutive repressors or promoters of adventitious rooting, but rather act in a context-dependent manner to control developmental competence for adventitious root formation. Light is a key regulator of adventitious root initiation, in part through modulation of auxin signalling components (Gutierrez et al., 2009). Whether SMXL3/SMXL5 function directly within light-responsive pathways or

indirectly through developmental states associated with etiolation remains unresolved. Nevertheless, the contrasting phenotypes observed here suggest that SMXL3 and SMXL5 may restrain auxin-driven adventitious rooting in light-grown hypocotyls, while being required to enable or sustain auxin responsiveness during the inductive phase following etiolation.

Unlike adventitious root formation, SMXL4 superclade proteins redundantly control RSA in an EAR motif-dependent manner. RSA is highly plastic, adapting to environmental cues such as gravity, stress, salinity, water and nutrient availability, *inter alia*. Root responses typically involve changes in primary and lateral root growth kinetics, reflecting a regulated balance between the two (Yalamanchili et al., 2024). In the taproot system of young Arabidopsis, primary root growth usually dominates; however, under conditions like phosphorus or nitrogen deficiency, lateral root elongation is favoured (López-Bucio et al., 2002; Remans et al., 2006; Williamson et al., 2001). In *lrd3/dar2* mutants, primary root growth is reduced while lateral roots dominate due to loss of the auxin-responsive DA1-RELATED2 (DAR2) protein which controls phloem loading in root tips, causing altered allocation of resources from primary to lateral roots (Ingram et al., 2011). Likewise, overexpression of dominant-negative PDGLP1/2 weakens primary root growth and enhances lateral root development via changes in plasmodesmal transport (Ham et al., 2012). In double mutants, anchor and lateral roots often outgrow primary roots, with the SMXL3 EAR motif apparently playing a central role in maintaining primary root dominance. SMXLs may regulate phloem partitioning among sink organs, akin to *lrd3/dar2* and *PDGLP1/2* lines, where reduced primary root sink strength enables lateral roots to compete more effectively for phloem capacity. Wallner et al. (2017) proposed that short primary roots in double mutants result from impaired phloem capacity, while Ingram et al. (2011) suggested that limited carbohydrate delivery to the primary root may trigger an as-yet-unknown 'loss of apical dominance' signal, stimulating lateral root growth. Whether the shift from primary to lateral root growth in double mutants and *SMXL3 EAR* mutants merely compensates for reduced primary root growth, or whether SMXLs actively arrange source-sink relations within the root system, remains an open question. Nevertheless, *SMXL3^{EAR}* roots more closely resemble those of Col-0 than double mutants, suggesting that SMXL3 directly contributes to primary root dominance in an EAR motif-dependent manner.

Similarly, root waving also depends on the SMXL3 EAR motif. Waving is an oscillatory growth pattern controlled by gravity, touch and asymmetric auxin distribution (Nziengui et al., 2018). Directional auxin flow is mediated by plasma membrane PIN-FORMED (PIN) efflux carriers, whose polar localization is regulated through

phosphorylation via antagonistic effects of phosphatases and kinases (Grones et al., 2018; Michniewicz et al., 2007; Rashotte et al., 2001; Wisniewska et al., 2006; Zhou & Nussbaumer, 2004). In mutants with disrupted PIN regulation, such as *rcn1* (Garbers et al., 1996; Rashotte et al., 2001), *wag1,2* (Santner & Watson, 2006) or *Pin1At* overexpressors (Xi et al., 2016), roots show enhanced curling on NPA-supplemented plates or display NPA resistance. Intriguingly, while NPA reduces primary root length, it simultaneously promotes curling in *SMXL3^{ΔEAR}* lines, suggesting that SMXL3 may contribute to the stabilization of basal (rootward) PIN localization in an EAR motif-dependent manner.

The suppression of anchor root formation is also SMXL3 EAR-dependent. Auxin signalling is required for anchor root formation with anchorene modulating auxin transport and homeostasis by conjugation suppression (Jia et al., 2019; Ke et al., 2024). While *SMXL3^{ΔEAR}* lines and other combinations are NPA-sensitive but slightly insensitive to NF, these observations suggest that SMXL-mediated suppression of anchor root formation primarily involves auxin redistribution, potentially operating in parallel with or partially independent of, carotenoid-derived pathways. These findings collectively point to a role of SMXL3, SMXL4 and SMXL5 in modulating auxin-dependent root developmental processes, likely by influencing auxin redistribution and/or local accumulation. The strong dependence of RSA, root waving and anchor root suppression on the SMXL3 EAR motif further suggests that SMXL-mediated transcriptional repression may contribute to stabilizing auxin flow and developmental dominance relationships within the root system. How SMXL proteins interface with auxin redistribution and accumulation, or with phloem-mediated resource allocation, remains an important question for future investigation.

In conclusion, our findings indicate that SMXL3, SMXL4 and SMXL5 act largely redundantly but also in specific pairwise combinations, suggesting that developmental outcomes arise from combinatorial rather than strictly specialized SMXL functions. Multiple root phenotypes point to a role for SMXL proteins in modulating auxin-dependent processes, likely by influencing auxin redistribution or local accumulation within the root system. Also, SMXL3 exerts both EAR motif-dependent and EAR-independent functions, with EAR-dependent activity predominating in root-associated processes and EAR-independent roles contributing to shoot and embryo development. By analogy to other SMXL family members, these observations support a model in which SMXL proteins form regulatory hubs that may integrate transcriptional and non-transcriptional mechanisms to fine-tune plant development in a context-dependent manner.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *smx13-1* (SALK_024706), *smx14-1* (SALK_037136), *smx15-1* (SALK_018522), double mutant combinations and *SMXL4_{PRO}:SMXL4:YFP* and *SMXL5_{PRO}:SMXL5:YFP* complementation lines were kindly provided by Thomas Greb (Heidelberg University). Genotyping was performed as per Wallner et al. (2017). For the phenotyping of aerial parts, seeds were sown on ½MS medium (Merck-Sigma Group; Darmstadt, Germany) supplemented with 10 g L⁻¹ sucrose (Merck-Sigma Group), then 7-day-old seedlings were transferred to pots filled with Compo-Sana soil mixture (Compo, Münster, Germany). Plants were grown in Conviron chambers (Madison, USA) with a 16 h/8 h light/dark photoperiod (110 μmol m⁻² s⁻¹, 21°C/19°C, 65% RH), equipped with L14 LED sources (Valoya Oy; Helsinki, Finland), according to Gulyás et al., 2022. For root and adventitious root growth assays, surface sterilized and stratified seeds were initially sown on ½MS plates containing 10 g L⁻¹ sucrose. Then, 2-day-old seedlings were transplanted to plates supplemented with various chemicals. For adventitious root induction assays, freshly sown seeds were incubated in light for 12 h, then transferred to dark for 4 days. All Petri dishes were incubated under the same environmental conditions as mentioned above.

Chemicals

Norflurazon (NF; Merck-Sigma Group), *N*-1-naphthylphthalamic acid (NPA; Merck-Sigma Group), *rac*GR24 (OChemIm, Olomouc, Czech Republic), enantiopure (+)5-deoxystrigol ((+)5DS; OChemIm) and karrikin 2 (KAR2) were used at 1 μM. KAR2 was synthesized as per Flematti et al., 2007. Anchorene (A; [2E,4E,6E]-2,7-dimethylocta-2,4,6-triene-dial; Biosynth, Berkshire, UK) has been used at 20 μM (Jia et al., 2019). FluorA I./II. were synthesized and the structure has been validated by NMR spectroscopy following Pařízková et al., 2021. DMSO (Merck-Sigma Group) was used throughout to dissolve compounds.

Phenotyping

Soil-grown plants were randomized every 5 days. For rosette area, height and silique measurements, whole plants or collected siliques were photographed on the days indicated and analysed with ImageJ (Schneider et al., 2012). The day of primary inflorescence arrest (post-germination) was approximated as the day when the last terminal flower on the primary inflorescence opened (Walker et al., 2023). Branching numbers were counted on inflorescence arrest, and branches ≥5 mm were considered. Anthocyanin extraction followed Jeong et al. (2010), with modifications: 250 mg of frozen, pooled samples of 5 plants were incubated in 1 mL acidified methanol (Merck-Sigma Group; 1% HCl). Subsequently, equal volume of chloroform (Merck-Sigma Group) was added, and the absorbance was measured at 530 and 657 nm as per Mancinelli (1990). Root traits on vertical ½MS plates were analysed on the days indicated. VGR was assessed from plate images; other parameters were measured on specimens preserved in 70% ethanol. For collet identification, seedlings were stained with 0.05% toluidine blue (Merck-Sigma Group) according to Janková Drdová et al. (2019). Germination tests were performed on agar plates using sterilized, stratified seeds.

Complementation of *smx13* mutation

To create complementation lines, a parental vector was constructed in *pENTR4* (*ccdB*). Primer sequences are listed in

Table S2. First, a full-length, partially digested *SMXL3* (*At3G52490*) cDNA was cloned into the *NotI/EcoRI* site then a 3.1 kbp promoter fragment was inserted into the *NcoI/NotI* site. Next, a synthesized fragment consisting of YFP and *SMXL3* 3'UTR has been cloned into the *EcoRI/XhoI* site to generate *SMXL3_{PRO}:SMXL3:YFP*. For *SMXL3_{PRO}:SMXL3^{ΔCIP-N}:YFP*, a synthesized fragment containing deletions between positions 70–162 and 391–507 has been incorporated into the cDNA fragment by overlapping PCR. *SMXL3_{PRO}:SMXL3^{ΔD1}:YFP* and *SMXL3_{PRO}:SMXL3^{ΔD1Ala}:YFP* were generated by deleting the GDLNL motif or introducing point mutations (ADANA) via PCR then ligated into the *SapI/BsWI* sites. For *SMXL3_{PRO}:SMXL3^{ΔEAR}:YFP* and *SMXL3_{PRO}:SMXL3^{ΔEARAla}:YFP*, LDLNL deletions or ADANA mutations were cloned using the *NotI/BsWI* sites. Final constructs were recombined into *pGW501* (Nakagawa et al., 2007) with LR clonase (Thermo Fisher, Waltham, MA, USA), transformed into *Agrobacterium tumefaciens* GV3101 and used in floral dip transformation. T₃–T₅ homozygous transgenic lines were used for all experiments. Independent transgenic lines are denoted with letters enclosed in double quotation marks throughout all figures. The expression of transgenes in transgenic lines has been assessed according to Végh et al., 2017 (Figure S7).

Microscopy

Root architecture and hypocotyls were examined *in vivo* or after mounting in 70% ethanol using a Leica DMS1000 microscope (Leica Microsystems, Wetzlar, Germany). For FluorA treatments, 5-day-old seedlings grown on vertical ½MS medium with sucrose were incubated in liquid ½MS containing 2 μM FluorA I. or II. for 30 and 15 minutes, respectively; fluorescence was then detected immediately (Párizková et al., 2021). Torpedo-stage embryos for YFP detection were isolated as described by Wallner (2018). Silique and embryo samples were collected at 13–14 DPA, cleared in a 6:1 ethanol: acetic acid mix, washed several times in 96% ethanol and mounted overnight in modified Hoyer's solution (chloral hydrate: glycerol: water, 8:1:2) (Berleth & Jürgens, 1993). Embryo imaging was done using Nomarski optics on an Abberior Infinity Line STED/confocal microscope (Abberior, Göttingen, Germany). YFP signal in roots and embryos (excitation/detection: 514/520–600 nm) and FluorA I./II. (excitation/detection: 488/490–600 nm) were detected with Leica TCS-SP8 and Abberior STED/confocal microscopes. Confocal images and z-series were deconvolved using Huygens Essential software (v25.04; Scientific Volume Imaging, Hilversum, The Netherlands).

Statistical analysis

ANOVA and *post hoc* comparisons (Games–Howell tests) and Kruskal–Wallis non-parametric test ($P < 0.01$) with Dunn–Bonferroni tests were performed with OriginPro (OriginLab Corporation, Northampton, MA, USA) and R (version 4.4.1; The R Foundation). Graphs were generated with OriginPro (OriginLab Corporation). Outlier data are shown as individual points. On box-plots, height delimits 25%–75% of data range. '●' symbols represent the individual data values, solid lines indicate the median, and '□' indicates the mean. The whiskers delimit ± 1.5 times the interquartile range.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

All data that support the findings presented in this article are available within the manuscript and the accompanying Supporting Information.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Distribution of adventitious roots (ADVs) along non-etiolated and etiolated hypocotyls and additional images. (A) Additional images: Morphology of non-etiolated hypocotyls and the collet region of 14-day-old *smxl3*, *smxl4* and *SMXL3_{PRO}:SMXL3* (*smxl3,5*) 'd' seedlings grown vertically on ½ MS agar plates with sucrose. Plants were mounted in ethanol. Bar = 0.5 cm. (B) Additional images: morphology of etiolated hypocotyls of 14-day-old *smxl3*, *smxl4* and *SMXL3_{PRO}:SMXL3* (*smxl3,5*) 'd' seedlings grown on vertical ½ MS agar plates with sucrose. Plants were kept in the dark for 4 days, then transferred to light for 10 days. Bar = 0.5 cm. (C) Distribution of ADVs along non-etiolated and etiolated hypocotyls of 18-day-old seedlings. In light-grown (non-etiolated) seedlings (left subpanels, labelled 'Light'), ADV numbers were quantified across five sections of *smxl3,5* hypocotyls. In dark-grown (etiolated) seedlings (right subpanels, labelled 'Dark'), the etiolated hypocotyl regions were divided into five segments, while the non-etiolated portion (emerging after the transfer of plates from dark to light) was analysed as a separate section. The x axes show the percentage of ADVs emerged in each section; y-axes denote hypocotyl length intervals (0–20%, 21–40%, 41–60%, 61–80%, 81–100%). Data represent ADV counts from 80 individual seedlings. (D) Spontaneous ADV formation in *SMXL3_{PRO}:SMXL3^{ΔCIP-N}*, *SMXL3_{PRO}:SMXL3^{ΔD1}*, *SMXL3_{PRO}:SMXL3^{ΔD1Ala}* and *SMXL3_{PRO}:SMXL3^{ΔEARAla}* complementing lines in *smxl3,5* background. Ratio of ADVs formed in non-etiolated hypocotyls is presented. The proportion of plants forming 0 to 4 ADVs is indicated by different colours. Plants were grown vertically on ½ MS agar plates with sucrose. Asterisks (*) indicate significant differences from Col-0 control; hash symbols (#) indicate differences from *smxl3,5* control plants; ns.: significantly not different from *smxl3,5* (4–6 biological replicates with 12 plants per replicate; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test). (E) Ratio of ADVs in etiolated hypocotyls 10 days after light exposure. Plants were grown on vertical ½ MS agar plates with sucrose. The proportion of plants forming 0 to 5 ADVs is indicated by different colours. Asterisks denote significant differences from Col-0 (mean $\pm$ SD; 4–9 biological replicates with 12 plants each Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).

Figure S2. Root responses to NPA and NF treatments and additional images. (A) Additional images: root morphology of 14-day-old *smxl3*, *smxl4* and *SMXL3_{PRO}:SMXL3* (*smxl3,5*) 'd' plants grown on vertical ½ MS agar plates with sucrose. Plants were mounted in ethanol. Bar = 2 mm. (B) The curly root phenotype of

14-day-old *SMXL3_{PRO}:SMXL3^{1EAR}* (*smxl3,5*) 'a' plants grown on vertical ½ MS agar plates supplemented with 1 µM NPA. Bar = 1 mm. (C) and (D) Root morphology of 14-day-old intact seedlings grown on vertical ½ MS agar plates supplemented with either 1 µM NPA (C) or 1 µM NF (D). Bar = 1 cm.

Figure S3. Rosette phenotypes of *SMXL3^{1CIP-N}*, *SMXL3^{1D1}*, *SMXL3^{1D1A1a}* and *SMXL3^{1EARA1a}* complementing lines in *smxl3,5* background. (A) and (B) Rosette morphology and area of 22-day-old plants grown under long-day conditions (16 h light/8 h dark). Rosette area has been assessed in 22-day-old plants grown under long-day conditions. Box-and-whisker plots with identical letters are not significantly different ($n = 60$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test). Scale bar = 5 cm.

Figure S4. Flowering time and height phenotypes of *SMXL3^{1CIP-N}*, *SMXL3^{1D1}*, *SMXL3^{1D1A1a}* and *SMXL3^{1EARA1a}* complementing lines in *smxl3,5* background and additional images. (A) Flowering time. Plants were grown under long-day conditions. Data were collected from 80 plants per genotype. Box-and-whisker plots with identical letters are not significantly different ($n = 80$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test). (B) Plant height at inflorescence arrest (IA). Height was measured on the day the terminal flower opened. Plants were grown under long-day conditions. Data were collected from 60 plants per genotype. Box-and-whisker plots with identical letters are not significantly different ($n = 60$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test). (C) Morphology of 57-day-old adult plants grown under long-day conditions. Scale bar = 5 cm. (D) Branching phenotypes of *smxl3*, *smxl4*, *smxl5*, *SMXL3_{PRO}:SMXL3* (*smxl3,5*) and *SMXL3_{PRO}:SMXL3^{1EAR}* (*smxl3,5*) plants. Plants were grown under long-day conditions. Data were collected from 64 plants per genotype on the day of IA. Primary branch number includes the primary shoot. The x-axis indicates branching order (1st to 5th) in each genotype. Box-and-whisker plots with identical letters are not significantly different. Lowercase, uppercase and numerical labels indicate statistically distinct groups for primary, tertiary and quaternary branches, respectively ($n = 64$; Kruskal–Wallis non-parametric test, $P < 0.01$; Dunn–Bonferroni test).

Figure S5. Double mutant combinations accumulate anthocyanin. (A) Anthocyanin content of 15-day-old rosettes. Plants were grown under long-day conditions. Data were collected from 50 plants per genotype. Box-and-whisker plots with identical letters are not significantly different ($n = 50$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test). (B) and (C) Anthocyanin content in leaves of 30-day-old rosettes and mature inflorescences (stem segments). Plants were grown under long-day conditions. For each plant line, the two data groups correspond to measurements plotted on the left and right y-axes, respectively. Data were collected from 50 plants per genotype. Lowercase and uppercase letters distinguish statistical groups for anthocyanin content in rosettes and stems, respectively ($n = 50$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test). Bar = 1 cm (C).

Figure S6. SMXL3, SMXL4 and SMXL5 contribute to silique development. (A) Morphology of green siliques at 13–14 days post-anthesis (DPA). Siliques were harvested from plants grown under long-day conditions and cleared in modified Hoyer's solution. Scale bar = 0.2 mm. (B) Siliques of *smxl3,4* are substantially shorter, whereas *smxl3,5* siliques are wider than those of Col-0. The dimensional parameters of green siliques at 13–14 DPA are presented. For each plant line, the two data groups correspond to measurements plotted on the left and right y-axes, respectively. Box-and-whisker plots with the same letter are not significantly different. Lowercase and uppercase letters indicate statistical groups for silique length and width, respectively ($n = 64$; ANOVA, $P < 0.01$; Games–Howell multiple comparisons test).

Figure S7. Expression analysis of wild-type *SMXL3* and *SMXL3* mutant transgene variants in 8-day-old whole seedlings of Arabidopsis transgenic lines. (A) Transcript levels were assessed by qPCR according to Gulyás et al., 2022 and are relative to the *UBQ10* reference expression. $n = 3$.

Table S1. Extracted Angiosperm SMXL3 and SMXL9 sequences.

Table S2. Protein sequences and list of primer sequences used in this study.

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