Closing report of the OTKA project „Interaction of DLK2 and SMXL3 to regulate plant development and stress tolerance: friends or foes?”

Introduction

Strigolactones (SLs) are carotenoid-derived molecules bearing essential butenolide moieties that were originally described as chemical cues promoting seed germination of parasitic *Striga* species (Al-Babili and Bouwmeester, 2015; Soós et al., 2019). It became evident that SLs are involved in controlling a wide range of plant developmental processes, including root architecture, establishment of mycorrhiza, stature and shoot branching, seedling growth, senescence, leaf morphology and cambial activity). Cumulative evidence supports the idea that the DWARF14 α/β-fold hydrolase (D14) functions as a SL receptor (Hamiaux et al., 2012; Waters et al., 2012). The structural rearrangement of D14 proteins in the presence of SL enables the protein to physically interact with the F-box protein MAX2 and SMAX1-LIKE (SMXL) (D53 in rice; Jiang et al., 2013) heat shock family proteins SMXL 6, 7 and 8 (Wang et al., 2015; Soundappan et al., 2015), which are transcription repressors. Therefore, strigolactones can promote gene expression by the targeted destabilization of the SMXL repressors.

In Arabidopsis, two paralogs of AtD14 have been identified (Waters et al., 2012b). One paralog, KARRIKIN INSENSITIVE2 (KAI2) was showed to confer insensitivity to karrikin (KAR), a butenolide-type germination stimulant from smoke water (Flematti et al., 2004). Although both AtD14 and KAI2 signaling pathways converge upon MAX2 and might employ similar mechanisms to transduce the signal, the two proteins regulate separate physiological events. Unlike AtD14, KAI2 genetically interacts with the other members of the SMXL family (SMAX1 and SMXL2), which redundantly regulate SL- and KAR-related gene expression (Stanga et al., 2013). KAI2 is required in Arabidopsis primarily for seed germination, normal seedling photomorphogenic responses, and leaf development (Waters et al., 2012; Soundappan et al., 2015), while in rice KAI2 is essential to perceive symbiotic signal needed for mycorrhizal association (Gutjahr et al., 2015). This functional divergence suggested that KAI2 is a component of an SL-independent signaling pathway that perceives a hypothetical butenolide ligand, hereafter termed as KL (for KAI2 ligand; Conn and Nelson, 2015), which is neither SL nor karrikin.

Very little is known about DWARF14-LIKE2 (DLK2), the third member of the DWARF14 protein family, to which only minor physiological role has been assigned as yet. Arabidopsis *dlk2* mutants in Col-0 background exhibit normal seed dormancy, photomorphogenic responses, and branching phenotypes (Waters et al., 2012), however, the mutants have narrow leaves and increased stem diameter. Interestingly, DLK2 overexpressing lines have broad leaves and elongated hypocotyls (Bennett et al, 2016; Végh et al., 2017). *DLK2* gene expression was recognized as a karrikin-responsive transcript in germinating lettuce achenes (Soós et al., 2012) and Arabidopsis seedlings (Waters et al., 2012b). *DLK2* is upregulated through the action of MAX2 or KAI2 in seedlings after SL or karrikin treatments, and its normal expression is highly dependent on light conditions and PIFs (PHYTOCHROME INTERACTING FACTORS; Végh et al., 2017). This butenolide-dependent expression has been hypothesized to be a negative feedback system in which DLK2 plays a role as a SL/KL metabolic enzyme (Scaffidi et al., 2013) or as a butenolide-sequestering molecule that fine-tunes butenolide levels. A detailed Differential Scanning Fluorymetry test, a hydrolysis assay and genetical evidences demonstrated that DLK2 does not bind nor hydrolyze natural (+)5-deoxystrigol ((+)5DS), and weakly hydrolyzes non-natural strigolactone (-)5DS, therefore DLK2 cannot be regarded as a SL/KAR receptor (Végh et al., 2017).

The known D14 family-related pathways converge at MAX2 and diverge at the level of SMXL-family proteins. Intuitively, the paralel evolution of SMXL and DWARF14 family proteins suggests that the weakly characterized members of the SMXL/D53 family, SMXL3, -4 and -5 might be co-opted by DLK2. SMXL4, originally referred to as AtHSPR (Arabidopsis thaliana HEAT SHOCK PROTEIN-RELATED), plays a role in abiotic stress responses (Yang et al., 2015) and displays a vascular bundle-specific expression (Zhang et al., 2014), as does DLK2 in elongating stem segments. It was shown recently that *smxl3,4,5* triple mutants are embryo lethal, *smxl3 smxl4* and *smxl4 smxl5* double mutants display severe carbohydrate accumulation and phloem transport deficiencies (Wu et al., 2017) and SMXL3, -4 and -5 are essential for phloem formation (Wallner et al., 2017). In SMXL3, -4 and -5, the RGKT motif needed for MAX2-mediated protein degradation of D53/SMXL7 is absent, suggesting that these proteins may not be degraded through MAX2 (Soundappan et al., 2015). These proteins act independently of MAX2 signaling, and SMXL5 is not degraded upon rac-GR24 application (Wallner et al., 2017). Taken togetherl, these findings clearly demonstrate that DLK2 represents a divergent member of the DWARF14 family, and, as a consequence of the paralel neofunctionalization of the two protein families, it is to be considered that DLK2 protein interacts with SMXL3,4,5 in a MAX2 independent manner. A preliminary BiFC assay in *Nicotiana benthamiana* leaves with DLK2 and SMXL3 split-YFP fusion proteins showed that the two proteins interact *in vivo* and this finding served as a good starting point to conduct experiments in the presented project.

Project report

A crucial task at the beginning of the project was to design and construct the plasmids required to generate the transgenics for functionalization tests. To generate barley CRISPR KO lines, we first cloned DLK2 and SMXL3 sequences and used the CRISPOR program to design the constructs. We transformed immature barley embryos using the established Agrobacterium-based transformation. Since the frequency of proper (few off-target events/ frameshift mutations) transformants is low, and the procedure is extremely time-consuming, we grew barley donor plants with a 3-4 month offset. We also prepared the Y2H vectors using pGADCg and PGBKCg plasmids. The Y2H system we used is based on the Matchmaker Gold procedure with few modifications. We prepared both N and C-terminal versions of plasmids encoding DLK2 and SMXL3 proteins, as well as the catalytic triad mutant of DLK2, to eliminate possible steric effects. The Y2H experiments revealed no interaction between the two proteins in any of the configurations tested. The most likely reason might be that DLK2 requires an endogenous ligand for interaction with SMXL3, as observed in paralogous systems (D14 vs. SMXL7). Additionally, it is conceivable that proteins playing a "bridge" role are also involved (similar to the SCF complex), as observed in paralogous proteins. We attempted to add plant extracts (ethanol, water, and ethyl acetate leachates) to the yeast culture medium, in the hope that the presumed endogenous ligand will induce the interaction of the two proteins. Unfortunately, we could not observe interaction in the presence of these extracts. Next, we repeated the pilot BiFC experiments with extended variety of the configurations and with mutant versions. The results are in full accordance with the findings from the preliminary experiments. More specifically, the C-terminal YFP:SMXL3 has been shown to interact with DLK2, while the construct harboring the N-terminal did not. The catalytic triad mutant version of DLK2 also failed to interact with SMXL3, indicating the presence of a ligand, thus explaining why the Y2H experiment was unsuccessful in the absence of an endogenous ligand. The highly conserved domain structure of SMXL3 predicts that SMXL3 is involved in protein-protein interactions, in line with the observation that the N-terminal truncated SMXL3 could not establish interaction with DLK2. To explore this phenomenon and to map the functional domains of the SMXL family, we created mutant versions of SMXL3. These mutants were used to complement *smxl3 smxl4* and *smxl3 smxl5* double mutants (since the function of these proteins is redundant, a strong phenotype is only observed in double mutants; triple mutants are

seedling lethal). We mutated the highly conserved regions of the N-terminal ClpN motif, the GDLNW motif (predicted to be responsible for interactions with MAX2 or other F-box related proteins), and the LDLNL motif in the C-terminal, where the EAR motif is located (Soundappan et al., 2016). Transformation and selection have been completed in the first year. In paralel, we have obtained plasmids required for FRET, Co-IP, recombinant protein production, promoter analysis, DLK2 expression induction and complementation tests; most of the constructs were completed in the first year. For Co-IP, we prepared 35S:SMXL3:HA vs. 35S:DLK2:GFP (and vice versa) constructs (, which were transiently transformed into protoplasts and *Nicotiana benthamiana* leaves. Stable transgenic lines were also generated, containing cassettes consisting of 35S:SMXL3:HA vs. 35S:DLK2:GFP, as well as pSMXL3:SMXL3:HA vs. pSUPERR:DLK2.GFP estradiol-inducible vectors. For FRET analysis, we prepared stable pSMXL3:SMXL3:YFP vs. p35S:DLK2:CFP transgenics. Towards the end of the first year, we also completed the construction of GUS and LUC vectors required for promoter analysis, containing various combinations of DLK2 promoter fragments, DLK2 UTR regions and cDNA (a total of 18 combinations). For the expression of recombinant proteins, we constructed vectors based on the pET (pET28c+ and pETARA) plasmids. DLK2 protein production was relatively hassle-free, while with SMXL3 we encountered difficulties (very low protein levels), likely due to its complex tertiary structure.

In the second year, we continued on with the inbreeding of CRISPR barley lines targeting DLK2 and SMXL3 genes. Unfortunately, we did not succeed; regenerating plants that survived on selective media either died or we were not able to detect the mutation in the target genes. In contrast, the DLK2 overexpressing construct, which was created in parallel and served as a positive control, worked appropriately indicating that the problem likely stemmed from the CRISPR construct (pHUE411). We replaced our CRSIPR plasmids with an alternative system (pBRACT vectors) with enhanced and plant-optimized Cas9. Unfortunately, our efforts with these enhanced vector systems also failed and we were not able to generate KO barley plants.

To further elaborate the proposed interaction between DLK2 and SMXL3 *in vitro* and *in vitro*, we tested a system in which a potentially obligatory endogenous ligand is present and could facilitate the interaction of the two proteins. For this purpose, we added ethyl acetate-based plant extracts to the culture medium (ethyl-acetate is widely used to extract SL-related apocarotenoids from plant tissues). Simultaneously, we conducted experiments using high-purity SL derivatives (e.g. 5-deoxy-strigolactone enantiomers) and SL-mimicking analogues (*rac*GR24). Unfortunately, we could not detect any interaction between DLK2 and SMXL3 in any of the configutrations tested either by Y2H or Co-IP. Since it has been suspected that the canonical interaction between D14 and SMXL 6,7,8 family could be surpassed by promiscuity (e.g. D14 vs. SMAX1/SMXL2; Oláh et al., 2021), we tested the interaction of SMAX1, SMXL3, and SMXL6 with D14, DLK2, and KAI2 proteins. These experiments revealed that in the presence of SL, D14 interacts with both SMXL3 and DLK2. This phenomenon may explain why *d14* mutants display MAX2-independent phenotype. We conducted FRET experiments to analyze the *in vivo* interaction in protoplasts transfected with p35S:DLK2:YFP and p35S:SMXL3:YFP constructs. The results indicated that the interaction between the two proteins is transient and weak. The absence of strong interaction can be attributed not only to the lack of the ligand or potential additional partner(s) but also to the difference in spatial expression. To test this, we created pDLK:DLK2:CFP pSMXL3:SMXL3:YFP (*smxl3 smxl5*) lines, which clearly demonstrated that DLK2 is expressed in the root cortical cells and root cap, while SMXL3 is expressed in the protophloem cell layer. Under normal conditions, no overlap has been observed between the two expression patterns. However, upon treatment with 0.01 uM IAA, DLK2 expression has been rearranged and the CFP signal has been observed in phloem cells of young lateral root primordia. We concluded that the two proteins are typically

expressed in separate tissues, however, under certain developmental conditions, when DLK2 expression is more relaxed, DLK2 protein expresses in protophloem cells. To demonstrate the physiological significance of the interaction, we created lines with promoter swap constructs. The complemented pSMXL3:SMXL3:YFP (*smxl3 smxl5*) lines containing the pSMXL3:DLK2:CFP construct exhibited significantly weaker growth, shorter roots and a phenotype reminiscent of the *smxl3 smxl5* double mutant phenotype. The promoter swap experiment confirmed that fine-tuning of SMXL3 protein levels in the protophloem cells took place through the regulation of DLK2 expression.

In the third year of the project, we evaluated the complementing lines in *smxl3 smxl5* and *smxl3 smxl4* mutant backgrounds to characterize the SMXL3 domain structure. We mutated the conserved regions of the N-terminal ClpN motif (referred as F1 lines), the GDLNW motif predicted to be responsible for interaction with MAX2 or other F-box proteins (F2 lines), and the EAR protein-binding LDLNL motif on the C-terminus (F3 lines). We were unable to restore the wt phenotype in F1 and F2 lines suggesting that the ClpN and GDLNW motifs are essential for proper function. Interestingly enough, not all phenotypic traits could be complemented in F3 lines, as anthocyanin accumulation and the root skewing phenotype were not complemented. This finding suggests that SMXL3 suppresses the expression of certain proteins through EAR motifs, but for the primary function of SMXL3, ClpN and GDLNW motifs are essential. Furthermore, we investigated the interactions of three known TPR proteins (TPR2, 3, 4) with SMXL3, SMXL5, and SMXL7 proteins in a Y2H system. None of them interacted with TPR3, while all three interacted with TPR4, and SMXL7 and SMXL3 interacted with TPR2. It is intriguing to consider that there might be a direct competition for protein partners between the paralogous proteins and TPR-SMXL interactions exhibit promiscuity, allowing evolutionarily diverged SMXL proteins to interact with other protein partners. Furthermore, it is interesting that the SMXL3 EAR mutant also interacted with TPR2 and TPR4, indicating the presence of alternative binding sites. It is known that gene expression could be considerably regulated by the mRNA untranslated regions (5' and 3' UTR). In the case of SMXL6, the G-quadruplex located in the 5' UTR and the associated JULGI proteins regulate mRNA stability. However, it is not known what extent the 3' UTR region of SMXL contributes to RNA stability, therefore we created complementing lines harboring constructs with or without 3' UTR. We found that the presence of the 3' UTR is essential to restore wild-type SMXL3 expression, and its absence results in short roots and slightly dwarfed plants in complemented lines.

During the second and third year, we made progress to understand the regulation of *DLK2* gene expression. LUC constructs driven by DLK2 promoter fragments were created, and wt and *htl-3* (Col-0 background KAI2 mutant) protoplasts were transfected. Our experiments revealed that the -98bp core promoter element is essential, while KAI2/karrikin-specific motifs can be roughly mapped to the region between -750 to -1180 bp. Using minimal 35S and native core promoter - DLK2 promoter fragment fusions, we finely mapped the karrikin-responsive (KR) motif to the -998 to -1042bp region. Additionally, we identified a suppressor region in the -1190 to -1270 region, which induces the inactivity of the KR motif.

The systems used in the first two years to investigate DLK2 - SMXL3 protein interaction (Y2H, Co-IP, BiFC) *in vitro* provided only weak evidence of direct interaction. The paralel evolutionary history of these proteins and the strong interactions observed in paralogous systems indicate that indirect interaction might take place between the two potential partners. The main obstacle to the physical interaction is primarily the different tissue expression pattern of the two proteins, however, there are cases where DLK2 expression pattern is altered and the signal (CFP) appears in protofloem cells also. Promoter swap constructs were introduced into *smxl3 smxl4*, *smxl3 smxl5* and *smxl4 smxl5* backgrounds. To obtain a comprehensive picture of the phenotypes the mutations caused, we subjected

each mutant line and thir combinations to comprehensive phenotyping. We showed that the phenotype of the single mutant lines (*smxl3-1*, *smxl4-1*, *smxl5-1*) did not differ from the wild type (Col-0). Contrary, double mutants have a short root phenotype, and *smxl3 smxl4* and *smxl4 smxl5* genotypes display a significant increase in lateral root formation. Furthermore, double mutants can be considered "longevity" mutants because they typically start bolting around day 40 (Col-0: day 21), and complete flowering in 70-90 days. Also, the mutants exhibit a remarkable reduction in height and rosette diameter and accumulate anthocyanins even under normal light conditions. The siliques of *smxl3 smxl4* are extremely short with 70-80% less viable seeds and aborted embryos. In contrast, *smxl4 smxl5* mutant seed lots have 10-20% aborted embryos at the heart stage, while the endosperm development is normal. Our investigation clearly indicated that SMXL3, 4, 5 proteins are functionally redundant and typically cooperate in pairs. With these results in mind, we evaluated the promoter swap experiment started the previous year. It became evident that the pSMXL3:DLK2 construct did not induce any phenotypic changes *in smxl3 smxl4* or *smxl3 smxl5* backgrounds. In contrast, we achieved a dramatic effect in *smxl4 smxl5* backgrounds, where only the SMXL3 protein is active. We managed to grow only four independent lines, as most of the transformants died during selfing (17 lines). The surviving lines are typically very slow-growing, start bolting around day 50-60, have an extremely small rosette diameter (40% of *smxl4 smxl5* rosette), and most of the seedlings die during growing ("soil lethal"). An intense anthocyanin accumulation has also been observed, and 80% of embryos aborted at the late globular stage. Plants carrying the promoter swap construct exhibit substantially higher number of secondary and tertiary branches suggesting that SMXL3 interacts with the strigolactone signaling pathway under specific conditions, thereby suppressed branching. It is known that the DLK2 protein evolved through neofunctionalization within the D14 strigolactone receptor family, and its expression is highly dependent on karrikin signaling and the presence of SMAX1/SMXL2, as well as KAI2 proteins (Waters et al., 2012; Oláh et al., 2019). It is conceivable that in the absence of SMXL4/5 in the promoter swap lines, DLK2 suppresses specific points in the strigolactone signaling pathway. An interesting phenomenon is that this suppression occurs not in the primary (where SL exerts its effects), but in the secondary/tertiary meristems. We also investigated the expression of genes involved in SL-dependent anthocyanin accumulation. We found that the expression of these genes (e.g., PAP1, MYB32) significantly increased in the promoter swap lines, consistent with the observed anthocyanin accumulation. These findings suggest - in addition to the high mortality rate of young seedlings - that SMXL3 degradation is enhanced in the presence of DLK2, leading to a remarkably low fitness in the promoter swap lines. Furthermore, we observed a significant decrease in floem-specific DLK2:CFP fluorescence in promoter swap lines in *smxl4 smxl5* background, indicating protein degradation. Our findings clearly confirm that the phylogenetically predicted co-evolution of DLK2 and SMXL3/4/5 manifests as a negative interaction between these two families (both parties undergo degradation) in the abscence of functional SMXL4 and 5. Therefore, we have answered the question posed in the title of the project, namely, whether DLK2 and SMXL3 are friends or foes: they are foes. The writing of a comprehensive paper on these results is underway.

The strong, auxin-responsive expression of DLK2 in root cortex (GUS, GFP, CFP) suggested a potential role for DLK2 in root architecture. To further investigate this phenomena, we grew wt and *dlk2* seedlings under conditions that alter the general structure of the root system, such as low P and N content, sucrose-free medium, strigolactone-containing medium, or the presence of root-endophytic fungi (*Piriformospora indica*). There were no significant differences in the development of roots co-cultivated with *Piriformospora* (wt, *dlk2*, pDLK2:GUS, pDLK2:DLK2:GFP) compared to controls. However, slight increase in root hair number and lateral branching were observed on low P and N content media. We demonstrated earlier that DLK2 expression is highly dependent on KAI2 receptor, a central

piece of a signaling paralogous fro D14 signaling. To gain a better understanding of DLK2 expression regulated by karrikins, mutant plants related to the karrikin receptor KAI2 were examined. For this purpose, two *kai2* mutants were isolated by sequencing from the *Brachypodium distachion* TILLING population generated by USDA and Princeton University in collaboration with the University of Western Australia (Dr. Mark T. Waters) and the Technische Universitat München (Dr. Caroline Gutjahr). The complex experimental setup revealed that KAI2 is essential for establishing mycorrhiza and allowed us to identify further KAI2 and D14-specific transcripts. DLK2 has three sub-paralogs present in the Brachypodium genome, with non-overlapping expression and high tissue specificity. Only DLK2c expression was found to be karrikin and strigolactone-dependent, while DLK2a and DLK2b did not respond to karrikin treatment. DLK2c expression highly correlated with root mycorrhization, which is in line with the recent observation that DLK2 plays a role in restricting the onset of mycorrhiza in tomato. Additionally, we searched for expression patterns highly similar to DLK2. The investigation revealed few novel KAI2/karrikin-dependent genes which coexpress with DLK2, such as ODPR5, which is involved in jasmonate biosynthesis, and WRKY28/71 transcription factors which are indispensable for ABA signaling and biotic stress responses. The results were published in The Plant Journal (Meng et al., 2022).

Furthermore, we examined the possible role of the paralogs of proteins related to SL biosynthesis in SL, karrikin, or even DLK2 (hypothetical) ligand synthesis. The ligands for KAI2 and DLK2 are still elusive. It was intriguing to consider whether the paralogs of the D27 beta-carotene isomerase, which catalyses the first dedicated step in strigolactone biosynthesis, might contribute to the synthesis of these enigmatic compounds. Therefore, we performed a comprehensive analysis with the closest paralog of D27, D27-LIKE1 which has been functionally diverged from D27. Although we've found that D27 and D27-LIKE1 is not related to KAI2 and DLK2 ligand biosynthesis, surprisingly, we demonstrated that the enzyme is involved in an auxiliary SL biosynthesis shunt. Due to neofunctionalization, D27-LIKE1 also acts as a violaxanthin isomerase, therefore directly contributes to the regulation of ABA biosynthesis. Our findings definitely opened new avenues in the research of how strigolactones and ABA are interfering even at the level of biosynthesis. These findings have been published in The Plant Journal (Gulyás et al., 2022) and in Journal of Experimental Botany (Tolnai et al., 2023, accepted).

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