Final Report (project ID: 114567)

The first year of the project started with the synthesis of the compounds needed for the future experiments. The synthesis of TMB, D-OH and their azido derivatives were optimized, elaborated and published (Pošta et al, 2016, Tetrahedron). We found that strigolactone degradation products can be modified in order to be suitable for photoaffinity labelling procedures, without harnessing the biological activity.

In paralel, seeds of strigolactone-related mutant lines were obtained through the European Arabidopsis Stock Centre (NASC), except *dlk2-4* (in Ler background), which was isolated from the Cold Spring Harbor Ds insertion lines collection (ET7593). Genotyping of *dlk2-4*, *dlk2-2* (SALK_068313C) *dlk2-3* (SALK_026193C), *d14-1* (WiscDsLoxHs137_07E), *kai2-2* (SGT6839), *max2-1* and *max2-2* lines used in this study were genotyped as described and were brought to homozygosity. To generate DLK2 overexpressing lines (in Ler, Col-0 and dlk2-2 background) with 6xHA tag (2x35S:cDLK2:6xHA), a secondary 35S promoter has been added to pCAMBIA1305. DLK2 cDNA coding sequence has been reverse transcribed from Ler total RNA, and DLK2 cDNA with a 6XHA tag was inserted into pCAMBIA1305-2x35S. To generate DLK2 mutants with altered binding site conformations (2x35S:Δ2+3cDLK2, 2x35S:Δ1+4cDLK2 and 2x35S:Δ1+5cDLK2), sequence verified gBlocks were inserted into pCAMBIA1305-2x35S. To prepare DLK2pro:GUS (in Ler and Col-0 background), a 1023 bp long genomic sequence including the promoter and 5' UTR has been amplified. For DLK2proDLK2intron:GUS, a 2361 bp long sequence including the 1023 bp promoter and the first exon and intron of DLK2 were PCR amplified from Ler genomic DNA and recombined into the pGWB533 GATEWAY binary vector. For DLK2pro:DLK2:sGFP, the same 1023 bp long promoter region used for GUS contructs and the genomic coding sequence of DLK2 has been amplified and recombined into pGWB405 vector. For 35S:sGFP:cDLK2, DLK2 cDNA has been amplified and inserted into pGWB505. For 35S:sGFP::cΔCatcDLK2, the amino acid components of the Ser-His-Asp catalytic triad have been replaced with Ala, the sequence has been synthesized (gBlocks), verified by sequencing and recombined into the GATEWAY compatibile pGWB505 plasmid. All Arabidopsis transformations were done by floral dipping, using GV3101 Agrobacterium strain. Transformants were selected on 1/2MS plates containing Hygromycin B. In parallel, an experimental system to use Controlled cDNA Overexpression Lines (COA) has been set up. We generated an Arabidopsis CDNA library, which unfortunately was not representative enough to be used for further applications.

Previously we employed a genetic screen in lettuce to find out what genes and network might lay behind TMB action. Now we extended the screen to more RIL lines to achieve a high resolution map of TMB hypersensitivity. In a screen for TMB insensitive and hypersensitive lettuce cultivars and species, we identified two accessions in the collection of UC Davis. Lactuca sativa 'Salinas' is insensitive, while Lactuca serriola is hypersensitive to TMB. A RIL (Recombinant Inbred Lines) population of 220 individual lines were tested for TMB response; germination data were collected and analyzed using the Cartographer QTL analysis package. We found two distinct QTLs on chromosome 2 and 3, 14 cM each.

In the second year of the project we obtained, crossed and selfed *dlk2*, *d14* and the novel *htl-3* (*kai2* in Col-0, a kind gift from David C. Nelson, UC Riverside) mutant Arabidopsis lines. Also, DLK2 overexpressing and complementation lines have been generated in Ler, Col-0 (*dlk2-2*), max2 and *d14 htl3 dlk2* triple mutant backgrounds. In paralel, four different types of GUS contructs (promoter length and intron variants) and

GFP lines have been made in different backgrounds. The hypocotyl elongation tests clearly demonstrated that KAI2 is epistatic to its paralogs and functions as a primary promoter of seedling photomorphogenesis. Most importantly, *dlk2* does not change the strigolactone (SL) sensitivity of the mutants, suggesting that DLK2 is not a receptor nor a hydrolysis enzyme for SLs. To test this hypothesis, we performed DSF (Differential Scanning Fluorometry) and hydrolysis assays with recombinant DLK2 protein. We demonstrated that DLK2 does not bind nor hydrolyze natural (+)5-deoxystrigol ((+)5DS), however, weakly binds and hydrolyzes the non-natural strigolactone (-)5DS, which acts as a KAI2 ligand.

In a separate experiment we found that DLK2 is upregulated in the dark dependent upon KAI2 and PHYTOCHROME INTERACTING FACTORS (PIFs), indicating that DLK2 might function in light signaling pathways. We showed that DLK2 is localized in the cytoplasm and in the nucleus and unlike its paralog proteins, DLK2 is not subject to rac-GR24-induced (SL analogue) degradation, suggesting that DLK2 acts independently of MORE AXILLARY GROWTH2 (MAX2); however, its transcriptional regulation is mostly accomplished through MAX2. Interestingly, DLK2 overexpressing lines (OE) show enhanced cotyledon elongation under low light conditions, and this effect was more pronounced in triple mutant background, where DLK OE promoted cotyledon expansion and suppressed the elongation of the axillary branches suggesting that DLK2 indeed acts independently of MAX2, however, the signal transduced by its paralogs is converging in MAX2. Furthermore, we compared the phenotype of *max2* and triple mutants and found that triple mutant does not phenocopy *max2* as expected on the basis of structural similarity, suggesting that DLK functions independently of MAX2. In conclusion, these data suggest that DLK2 represents a divergent member of the DWARF14 family. These findings were presented at the Strigolactone 2017 conference (Turin, Italy, March 2017; oral presentation) and published in Frontiers in Plant Science (Végh et al., 2017). To find DLK2-related phenotypes, we performed the GUS expression assay with four different contructs and found that DLK2 is expressing in leaf epidermis, root cortex, axillary buds and vasculature adjacent to the buds. We also demonstrated that DLK2 expression pattern is highly dependent on SLs and light conditions. We performed RNA-Seq analysis of wild-type, *dlk2* mutant and OE seedlings and found that only a handful of genes were affected by DLK2 and these genes are predominantly related to ABA and IAA pathways. DELLA proteins were suggested to physically interact with DWARF14 family proteins. Recent publications showed that this is not the case and D14-DELLA interaction is an artefact, therefore we did not test this finding. Instead, a phylogenetical analysis of SMXL proteins, the suppressors of SL signaling suggested that DLK2 co-opts SMXL3, 4 and 5. We started to test this hypothesis in the second year with co-IP, Y2H and BiFC *in planta*. We also moved forward with the validation of the RIL screen. The expression of an alpha/beta fold hydrolase, designated as N85 has been found to correlate with TMB insensitivity in Lettuce 'Salinas' plants. We set up a functionalization test in Arabidopsis using N85 overexpressing and GFP lines, as well as mutant alleles and proved that N85 is required for TMB insensitivity. Regarding the Induced Overexpression System in Arabidopsis (OAS), we obtained selfed Arabidopsis lines harboring *Lepidium crassifolium* library.

In the third year of the project we continued protein-protein interaction studies with proposed DLK2 partners. We found that DLK2 is not a receptor for strigolactones or karrikins and likely acts independently of MAX2 (Végh et al., 2017). The parallel neofunctionalization of the DWARF and SMXL family proteins also suggests that DLK2 acts independently of MAX2 and physically interacts with three members of the SMXL family, SMXL3, 4 and 5. A detailed BiFC assay with Nicotiana benthamiana leaves revealed that DLK2 interacts in vivo with these SMXL proteins, however, the position of YFP tag (N-terminal) on SMXLs compromises the interaction, suggesting that the domain (presumably

a ClpC) responsible for protein-protein interaction can be found close to the N-terminal. We also performed BiFC assay with mutant DLK2 versions, where the amino acid residues of the proposed catalytic triad were exchanged to Ala. The paralogs of DLK2, D14 and KAI2 are functional only with intact catalytic functions, which is a prerequisite for proper ligand binding and interaction with MAX2 and other SMXL proteins. We found that the loss of the proposed catalytic Ser does not compromise SMXL3,4,5 interaction, however, the loss of the complete catalytic triad causes folding errors (assessed by fluorescent thermal shift assay). We conclude that contrary to its paralogs, catalytic activity is not required for DLK2-SMXLs interaction. To further assess and confirm BiFC results, we performed Y2H assay with DLK2 and SMXL3. The well-studied D14-SMXL7 partners were used as positive controls; we found that DLK2 and SMXL3 are not, or weakly interact in yeast, proposing that a ligand or other protein partners are required to form a protein complex. Co-IP assay with 35S:DLK2:GFP vs. 35S:SMXL3:HA or 35S:SMXL3:GST (in stable transformants) also produced weak or equivocal signals; Co-IP with pDLK2:DLK2:GFP vs. pSMXL3:SMXL3:GST resulted in weak signal, suggesting either a technical failure, or, more likely, the mutual degradation of both partners upon physical interaction (this is the case with SMXL7 and D14, where only the ΔRKGT-motif mutant of SMXL7 can interact with D14). To further evaluate this scenario, we generated estradiol-inducible DLK2:HA lines in pSMXL:SMXL:YFP (dlk2-2) background. Another option is that the tissue specific expression of these proteins can hinder physical interaction. Indeed, SMXL3:YFP is abundant in root apical region (especially in protophloem) and vasculature (phloem), contrarily, DLK2:GFP is expressing in root cortex and absent in root apex and phloem. It seems that SMXL3 localization is not adjusted by protein degradation (Wallner et al., 2017). This is not the case with DLK2, where the expression pattern of pDLK2:GUS and pDLK2:DLK2:GUS did not match, suggesting that targeted proteolysis can fine tune the exact spatio-temporal pattern. We observed that in pDLK2:DLK2:GUS plants, GUS signal is absent in root apices where SMXL3 is abundant. We assume that analogously to D14-SMXL7, DLK2 and SMXL3 mutually promotes their degradation.

Regarding the Induced Overexpression System in Arabidopsis (OAS), we began to test selfed Arabidopsis lines harbouring Lepidium crassifolium library. So far more than 700 lines (bulks) has been tested on 10uM rac-GR24 plates, but unfortunately, no suppression of MAX2 activity (elongated hypocotyl under low light conditions on GR24-supplemented media) has been observed so far.

The proposed interaction of DLK2 and SMXL3 suggests that DLK2 might have a function in regulating SMXL activity, therefore, *dlk2* mutants could mirror or mimic the phenotypes observed in *smxl* double mutants (sugar transport and phloem loading deficiency, arrested root growth, anthocyanin accumulation, protophloem defects). We set up an induction system in which ectopic phloem cell differentiation can be induced in cotyledons by bikinin, a potent inhibitor of brassinosteroid signaling (Kondo et al., 2016). *smxl* double mutants are insensitive to bikinin treatment, so we checked the response of DLK2 to this agent in a study using wt, *dlk2-1*, DLK2OE, and *smxl3 dlk2-1* double mutants. Also, we optimized sugar measurements (HPLC) to assess whether sugar transport is compromised in dlk2 mutants.In the previous year we performed the transcriptome analysis of wt, *dlk2* and DLKOX seedlings using RNA-SEQ. This year we continued the transcriptomics study with the validation of the results in independent samples by real-time RT-PCR. The most responsive set of 35 genes were selected and we've found that qPCR results do not fit well with transcriptome data. It seems that RNA-SEQ significantly "overshoots" expression values, therefore the data cannot be used for transcriptome analysis. However, we managed to pick several ABA-related genes which are truly regulated (0,2-several fold) by DLK2, such as WRKY22, HAI1, HAI2, NAC32, ABI1 etc. We also moved along with the TMB metabolization project. DLK2OX lines in Lactuca serriola background were selfed and we possessed homozygous T3 generations in four independent lines. Unexpectedly, the

synthesis of stable isotope labeled or modified (azide labeled) TMB proved to be rather difficult, either the structure was compromised, or the yield was too low. After extensive optimization of the synthesis (Posta et al., 2017, 2018), we prepared enough modified compounds to carry out the experiments (UPLC/MS or GC/MS analysis of achene extracts treated with TMB).

In the fourth year we continued on with the VISUAL experiments where brassinosteroid signaling has been inhibited with bikinin (Kondo et al., 2016). We found that DLK2OX lines act similarly to smxl3,4,5 double mutant combinations in terms of ectopic phloem induction, indicating that DLK2 might inhibit brassinosteroid pathway through GSK3 – BES1. The VISUAL assay also showed that DLK2 and SMXL3,4,5 might act antagonistically. These results prompted us to continue with checking whether tissue specificity or a truly antagonistic relationship (where DLK2 and SMXL proteins degrade in the presence of each other) lies behind this phenomena. We generated pDLK2.cDLK2:GUS and pDLK2:cSMXL3 lines in *smxl3 smxl4* background. Apparently, pDLK2:cSMXL3 did not restore the *smxl3 smxl4* phenotype either due to a strict tissue specific localization of SMXL3, or that DLK2 promoted the degradation of SMXL3. We observed that in pDLK2.cDLK2:GUS and in pDLK2.cDLK2:GFP lines DLK2 signal randomly appears and co-localize with SMXLs in the lateral root tip and stem vasculature. We speculated that DLK2 might function in lateral root extrusion. CLSM analysis of the GFP signature in this tissue revealed that DLK2 is highly expressed right upon the emergence of the root tip and fades away as the elongation proceeds. We checked several conditions under which lateral root growth is promoted, e.g. sugar deprivation, or colonization of the roots with symbiotic-endophytic fungi, such as *Piriformospora indica*. We found that co-cultivation of pDLK2.cDLK2:GUS roots with P. indica promotes DLK2 expression in root tips which resonates well with a recent finding that DLK2 is highly expressed in the mycorrhiza-colonized roots of tomato (Ho et al., 2018). Another line of evidence is that KAI2 KO rice plants (*hebiba* mutant, Gutjahr et al., 2015) did not establish mycorrhiza. We showed that KAI2, the paralog of DLK2 is required for the proper expression of DLK2, suggesting that the orchestrated action of these two paralogs is critical for the plant-symbiotic fungi interaction. We also checked the sugar composition of *dlk2* mutant leaves and phloem sap using HPLC. We found that sugar signature of the mutant differs from the wild type and shows opposite characteristics to *smxl3,4,5* double mutant combinations.

We also moved on with the UPLC/MS or GC/MS analysis of seed extracts treated with tagged TMB, a potent germination inhibitor which structure is highly related to karrikins. We assumed that upon cross linking the azide-TMB to its protein partner we could identify its binding protein (or receptor), or by using a stable isotope labeled TMB, we could answer the question whether TMB is metabolized, and if so, the metabolite is responsible for the observed effect. Unfortunately, after a copious amount of trials we could not detect neither azide-TMB tagged proteins nor stable isotope labeled TMB metabolite in germinating lettuce achenes suggesting that TMB is not metabolized. We propose that experimentation with azide-tagged TMB requires more optimization, or TMB does not interact with its protein partners in a canonical (receptor-ligand) way (as suggested by the high concentration used to achieve germination inhibitory effect.