FINAL REPORT (OTKA 100763)

Genomics of the effect of smoke derived germination cues

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Transcriptome analysis of KAR1 and TMB – treated germinating lettuce achenes

Our previous expression studies on the effect of the active compound KAR1 on germination showed that the lettuce bioassay is more suitable for studying the KAR1 effect in the early germination phase than maize kernels. Therefore we designed a novel lettuce microarray on Agilent platform consisting more than 28000 features using of DFCI, Unigene and other NCBI sequence records. The sequences were analyzed and compared with the BLAT and OligoArray v2.1.3 softwares. The oligos were submitted to the Agilent eArray site (http://earray.chem.agilent.com/earray/; ID: 028289), and after filling up the <60 nucleotide length oligos with linker sequences, they were synthesized on an Agilent 44k slide.

For annotation, we used the available information from UniGene, TIGR and DFCI, complemented with the Arabidopsis information from ENSEMBL. We filtered the possible Arabidopsis orthologs, requiring at least 40% sequence identity and 60% query sequence coverage, and used the descriptions, cross-references, GeneOntology and KEGG information for further analysis.

The microarray design consisted of two biological replicates and two dye-swaps. For each biological replicate, RNA samples (1 µg) of three technical replicates were pooled. After 2 h and 10 h of treatment, the germinated and non-germinated achenes were indistinguishable and all 50 achenes were used for the RNA isolation. For the 24 h samples, the RNA of germinated and non-germinated achenes were isolated separately and equal amounts of their RNA were pooled. All the samples from control experiments (achenes germinated in water and kept in the dark) were compared to samples treated with 0.1 µM KAR_1**,** 0.1 µM KAR_1/5 µM TMB or 10 µM TMB.

Scanning was performed with Agilent High-Resolution Microarray Scanner; the detection of signal intensities and the grid adjustment were accomplished with the Agilent Feature Extraction Software. Genes with a fold-change ≥ 2 and a corrected p-value ≤ 0.05 were considered differentially expressed. The microarray data have been deposited in the GEO database (GSE34642).

At 2 h, there was no overlap between the KAR_1- and TMB-responsive genes. In contrast, simultaneous application of the two compounds resulted in changes in the expression of all individual KAR_1- and TMB-response genes and a few other genes, such as the BTF3-like transcription factor (DY981384). The data indicates that there are extensive differences in the scope and degree of the gene expression changes provoked by the two compounds applied alone, and there is an intermediate response when both compounds are present. KAR_1 treatment manifested at the gene level by the repression of two gene families, the 11S globulin and the 2S albumin genes. TMB treatment yielded the downregulation of several genes, including alcohol dehydrogenases, a wound induced protein, kaurene synthase, UDP-glucose glucosyltransferases, a phosphate translocator, and a putative WD-repeat protein. Only one gene, a phosphatase, showed induction above the two-fold cutoff.

At 10 h, there was an overlap between the genes upregulated by both compounds. Surprisingly, all three TMB-induced genes, LEA, photosystem Q(B) protein precursor and a hypothetical protein could be found on the list of KAR_1-induced genes. TMB treatment repressed a hypothetical protein gene, FAR1, oxidoreductases, and also downregulated the alcohol dehydrogenases and a putative wound-induced protein gene which were also affected at 2 h. KAR_1 treatment resulted in the prominent upregulation of an α/β fold hydrolase (SIGB, DY983731), an AP2/ERF transcription factor, histone H2B, and the distinct downregulation of the cysteine protease-3.

Inspection of the KAR_1 and TMB responsive gene lists at 24 h revealed a reciprocal effect of TMB (Figure 1), and a specific set of genes showed a contrasting expression pattern after the treatments with the two compounds. FUS3, HVA22, LEA, NCED genes (e.g. LsNCED2), globulins were repressed after KAR_1 exposure, and induced in TMB-treated samples. On the contrary, the abundance of an aquaporin and hypothetical protein transcripts increased with KAR_1 treatment, and decreased with TMB treatment. Several light-related genes, such as ELIP, HY5, chlorophyll a-b binding proteins etc., were induced by KAR_1 treatment, whereas others, including hypothetical proteins and ATFP6, were repressed.

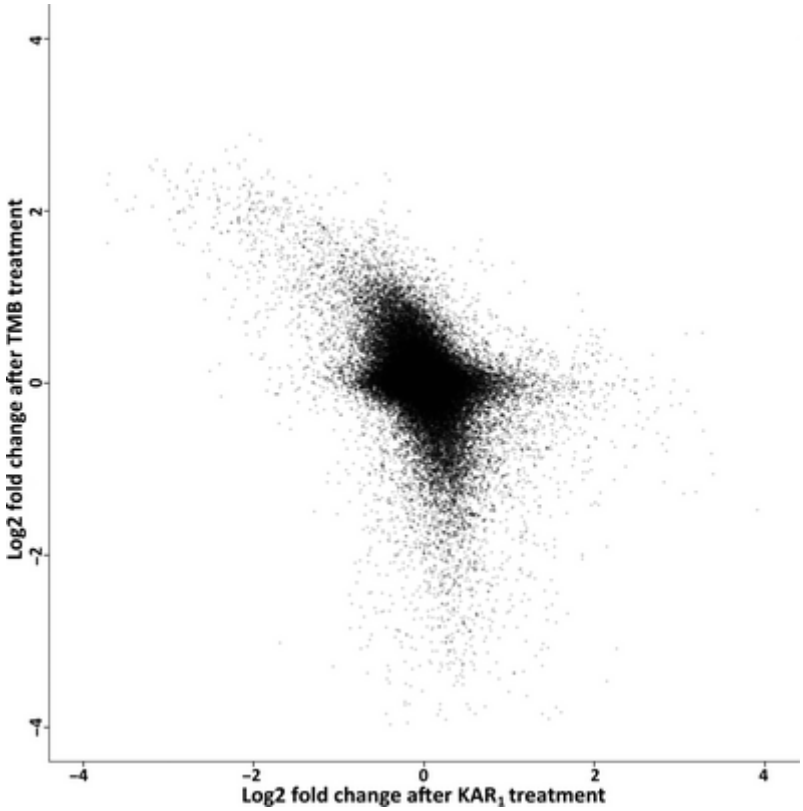

Figure1KAR_1 and TMB induce contrasting changes in the transcriptome of *Lactuca sativa* 'Grand Rapids' achenes after 24 h. Log2 fold-change data for each gene distinctly differentially expressed after KAR_1 treatment were plotted against their counterpart following TMB treatment.

Validation of the microarray study and the real-time PCR analysis of the expression of selected genes

To validate the microarray results, the differential expression for selected genes from all time points was corroborated using qRT-PCR. The 15 genes with distinct and characteristic expression changes were chosen, and several genes showing no expression change were also selected randomly for the validation process. The expression pattern observed in the microarray experiments was consistent with the genes analyzed by real-time PCR. The linear regression analysis showed a significant correlation between the two data sets, with $R^2 = 0.7498$.

KEGG and GO term analysis of the microarray data

The most pronounced GO terms, following KAR_1 or TMB exposure, were different and reflect fundamental differences induced by the two compounds (Figure 2). The presence of genes related to various biosynthetic and metabolic processes were robust in the

'TMB-down' list. A number of GO terms related to ABA were overrepresented in 'KAR_1-down' and 'TMB-up' list in a reciprocal manner. A similar contrasting pattern was observed in seed-related terms, where seed maturation, seed development and seed dormancy -related terms were abundant in 'KAR_1-down' and 'TMB-up' lists. Photosynthesis and light response-related terms were distinctly overrepresented in the 'KAR_1-up' list. Stress-related terms were also present in all lists.

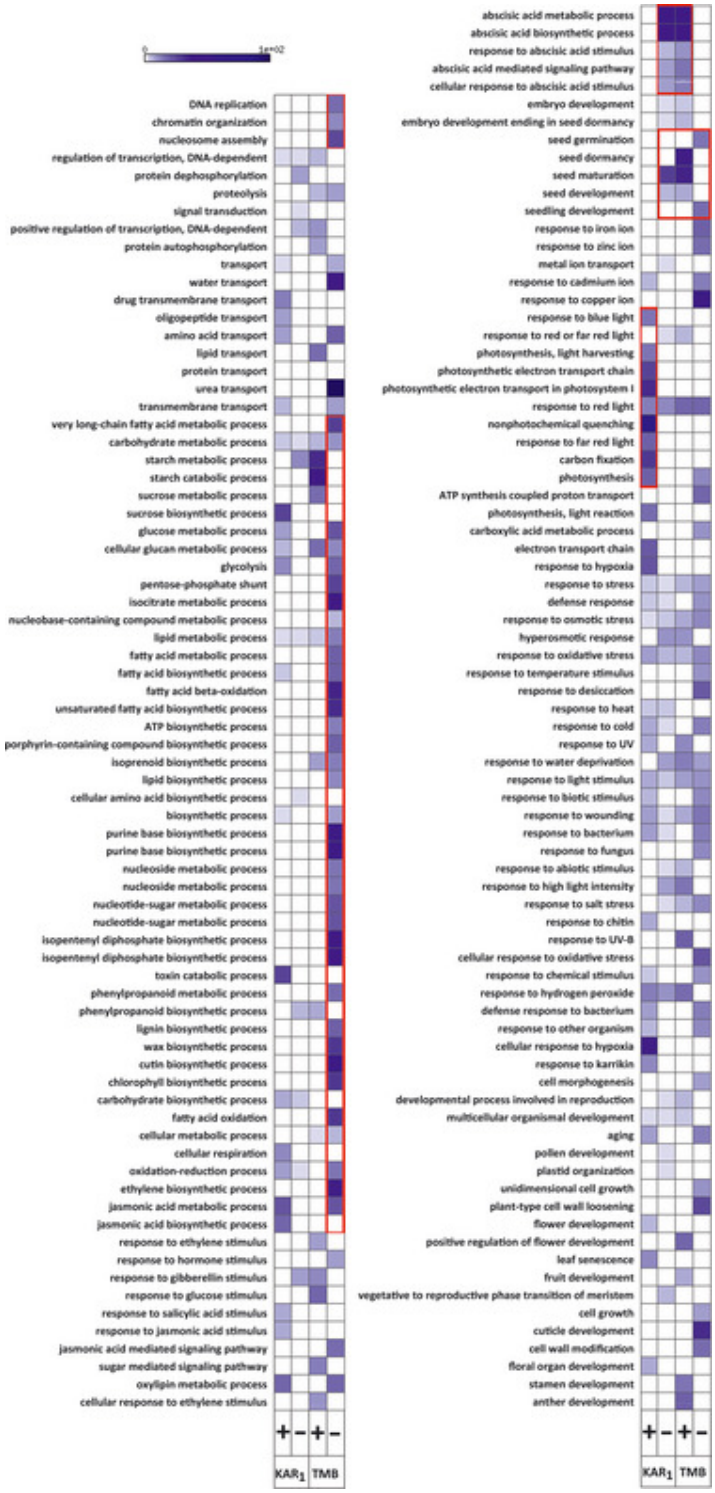

Figure 2: The list of Gene Ontogeny (GO) terms overrepresented in the group of genes up- and down-regulated after 24 h in karrikinolide (KAR_1)- or trimethylbutenolide (TMB)-treated *Lactuca sativa* 'Grand Rapids' achenes

(fold change ≥ 2 and corrected *P*-value < 0.05). Light colors indicate low representation; blue/deep blue colors show overrepresentation.

Physiological experiments and Schild regression analysis

To further evaluate the proposed differences between the KAR_1- and TMB-treated achenes germinated in the dark, the relevant parameters were extracted from the germination time-curve. Application of 0.1 µM KAR_1 and the combination of 0.1 µM KAR_1/1 µM TMB resulted in a significant increase in germination. The maximum percentage of germination (gMAX), the time to reach 50% germination (t_{50}), the mean germination time (MGT) and the AUC (area under the curve; a parameter that combines information on the above-mentioned values and germination uniformity values) all showed that KAR_1 alone, and a combination with a low concentration of TMB, not only increased germination rate, but also increased germination uniformity. In contrast, the application of 10 µM TMB and 0.1 µM KAR_1/10 µM TMB resulted in a significant decrease in the parameters evaluated, demonstrating that TMB is a strong germination inhibitor.
To characterize the proposed antagonistic relationship between the two compounds, Schild regression analysis was applied by which the effects of agonists and antagonists on the response caused by binding to an effector molecule can be studied (Figure 3). Each of the seven fixed-doses of TMB produced a decrease in the response magnitude and a rightward and parallel shift of the KAR_1 dose-response curves. At high concentrations of TMB (10 and 7 µM), KAR_1 was unable to fully overcome the negative effect of the TMB, while at a lower TMB concentration (1 µM), the KAR_1 effect could exceed that of the TMB. At the intermediate concentrations, however, the germination response depended on the TMB concentration demonstrating that, irrespective of the KAR_1 concentration used, germination can be significantly reduced by increasing the dose of TMB. The Schild plot for TMB against KAR_1 was nonlinear and the apparent slopes from the three experiments were significantly different from unity indicating that KAR_1 and TMB are not competitors.

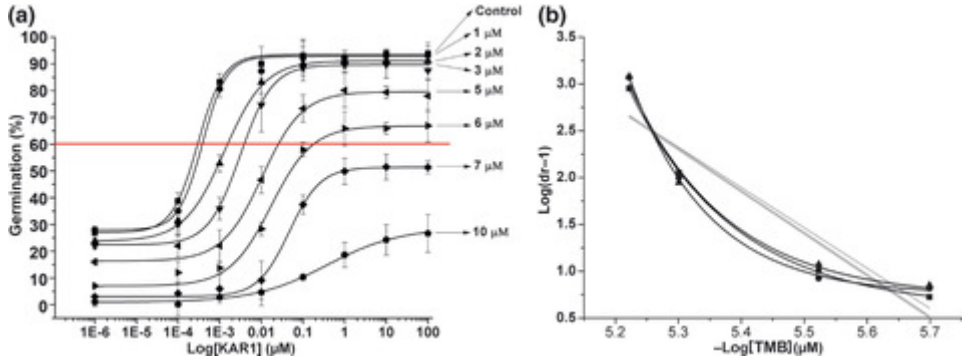

Figure 3: Schild regression analysis, indicating that karrikinolide (KAR_1) and trimethylbutenolide (TMB) are not competitors and TMB induces the germination response in a dose-dependent way. (a) Germination of *Lactuca sativa* 'Grand Rapids' achenes exposed to various concentrations of KAR_1 and TMB in a pairwise manner. The R_{50} value is marked with a red line and was calculated using the formula $R_{50} = (R_{max} + R_{basal})/2$, where R_{max} is the maximal germination percentage in the presence of KAR_1 and R_{basal} is the basal response in the absence of KAR_1. Each treatment consisted of three independent experiments (n = 50). Error bars represent SE of the mean germination percentage. (b) Schild regression analysis of the KAR_1–TMB interaction (black: nonlinear regression; gray: linear regression). The Schild plot was constructed by plotting log(dr − 1) {log([*A'*]/[*A*]) −1)} against −log{TMB} (−log[*B*]).

Germination response of KAR_1- and TMB-treated achenes to white, red (R) and far-red (FR) irradiation

The microarray data suggests that KAR_1 and TMB interact with light-dependent processes in Grand Rapids lettuce achenes. In order to distinguish between light and KAR_1/TMB responses, germinating achenes treated with the compounds were irradiated with continuous white light, FR and R light and the germinated achenes were counted after 24 h (Figure 4). Continuous white light or 1 h R fluence resulted in 96% and 100% germination, respectively. In contrast, 1 h FR fluence blocked germination and resulted in ~7% germination and a 3 h FR irradiation resulted in 0% germination. The negative effect of FR could be overcome by 0.1 µM KAR_1 in the presence of 1 µM TMB, while 10 µM TMB completely blocked germination, even when 0.1 µM KAR_1 was added. Treatment with white light could not overcome the effect of **10** µM TMB, whereas R irradiation could only partially do so.

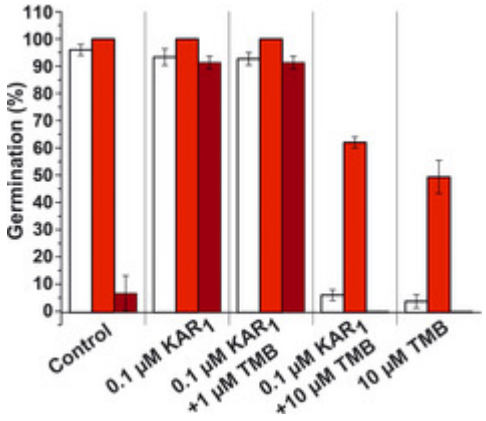

Figure 4: KAR_1 overcomes the negative effect of far-red irradiation, while red irradiation can only partially compensate for the effect of TMB treatment. The germination response of *Lactuca sativa* 'Grand Rapids' achenes after 24 h of treatment with 0.1 µM KAR_1, 0.1 µM KAR_1/1 µM TMB, 0.1 µM KAR_1/10 µM TMB and 10 µM TMB and irradiation with red or far-red light is shown. For the light-response assays, Petri dishes were irradiated with white fluorescent light (20 µmol m^{-2} s^{-1}; white bars), red light (5 µmol m^{-2} s^{-1}; red bars) or far-red light (2 µmol m^{-2} s^{-1}; dark red bars) for 1 h.

Leaching of KAR_1 and TMB from germinating lettuce achenes

We also hypothesized that the proposed antagonistic relationship between the two compounds is modulated by the different binding affinity of KAR_1 and TMB to their prospective effectors. To test this hypothesis, rinsing tests were carried out with both compounds and the germination response of the achenes in the dark was then recorded (Figure 5). KAR_1 could only be leached within the first 30 min of incubation and, thereafter, the germination rate increased sharply with incubation time reaching a plateau around 80 mins, after which the effect of KAR_1 was irreversible by rinsing the achenes. The TMB, however, could be leached at any time within the first 24 h, where the germination rate was similar to achenes kept in the dark. The simultaneous application of 0.1 or 1 µM KAR_1 along with 10 µM TMB revealed that after 24 h only the TMB effect was diminished by rinsing, and KAR_1 exerted its positive effect within the following 24 h. We also studied whether KAR_1 action could be reversed by TMB with the onset of germination. We found that a pulse treatment with 10 µM TMB resulted in the full reversal of the effect of 0.1 µM KAR_1 only within the first few hours, whereas, no effect was observed after 8 h.

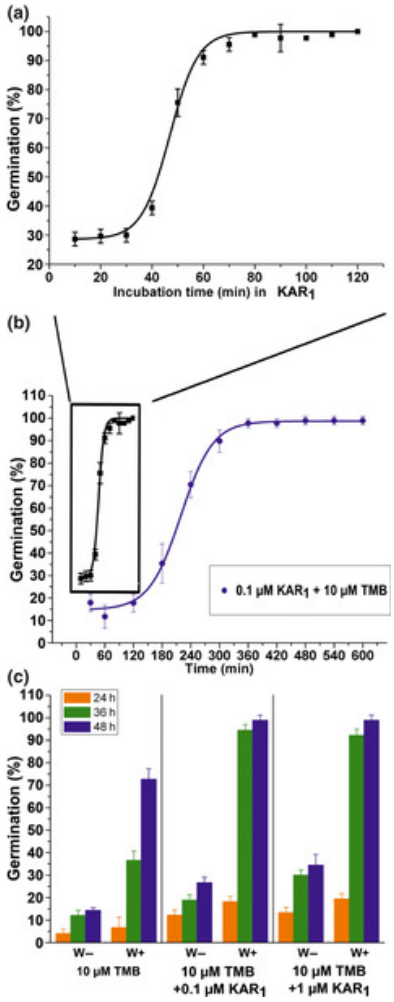

Figure 5. Leaching experiments with KAR_1 and TMB. (a) The effect of KAR_1 could not be diminished by rinsing in water after 70 min of exposure. *Lactuca sativa* 'Grand Rapids' achenes were germinated in 0.1 µM KAR_1 and the achenes were rinsed for 3 × 2 min in 200 ml of distilled water after 10–120 min. Germinated achenes were scored after 24 h.) The effect of KAR_1 could be reversed in the first 8 h of exposure with a 10 µM TMB pulse treatment. Achenes were germinated on filter paper moistened with 0.1 µM KAR_1, and after 30 min and every 1 h until 10 h the achenes were collected, drained and further germinated on filter paper moistened with a solution of 0.1 µM KAR_1/10 µM TMB. Germinated achenes were scored after 24 h. (c) The effect of TMB could be reversed at any time by rinsing the achenes in water. Achenes were treated with 3 ml of solution containing 0.1 µM KAR_1/10 µM TMB or 1 µM KAR_1/10 µM, germinated for 24 h and then rinsed with 27 ml of distilled water for 5 min. The excess solution was drained and the achenes transferred to filter paper and germinated for a further 24 h. Germinated achenes were counted 12 and 24 h after rinsing. W−, unrinsed; W+, rinsed.

Functionalisation tests with butenolide – responsive candidate genes

Based on the microarray study, we began the functionalization tests with the proposed master genes and gene networks selected on the basis of transcriptomics results. We chose a gene (former referred as 'SIGB', hereafter termed as DLK2) which showed a distinct upregulation after KAR_1 treatment. Furthermore, DLK2 (D14-like 2) is the paralogue of AtD14 and KAI2, the receptors for strigolactones (and the latter for karrikins) in Arabidopsis. Strigolactones share a very high structural similarity with karrikins, namely both cues have a functional butenolide moiety responsible for the function as a plant hormone. It was recently shown that strigolactones and karrikins act in parallel signalling pathways which are converging on the level of MAX2 F-box protein, a central player in strigolactone signalling. To date, no physiological role has been assigned for DLK2. We found that DLK2 is markedly upregulated in all parts of lettuce and Arabidopsis plants after KAR1 and strigolactone (as a GR24 racemic mixture or + and – strigol enantiomer pairs) application indicating that it is an overall response factor

for butenolides (both strigolactones and karrikins). We propagated three different *dlk2* mutants in Col-0 (2 from the SALK T-DNA insertion line collection) and Ler (1, transposon/insertion mutant from the Martienssen collection, Cold Spring Harbor Laboratories) background and generated a series of transgenic Arabidopsis plants overexpressing DLK2 or DLK2::His-tag to find out and study the role and the related phenotype of this subtle gene. We also made DLK2 overexpressing fusion contructs with HA-tag in *Lactuca serriola*, a good candidate model plant for strigolactone research. The plants were transformed at the UC Davis in vitro facility and the second generation is currently being selfed. Heterozygous L. serriola plants show a copy number dependent strong seed dormancy in the presence of strigolactones only under high light conditions suggesting that DLK2 might have a role in regulating photoblasticity in a strigolactone dependent manner. In parallel, we were interested in the spatio-temporal pattern of DLK2 expression pattern in Arabidopsis, so we generated a GUS construct with a 2.5 kb promoter region of DLK2 (OP). Surprisingly, this fragment resulted in no GUS activation suggesting the presence of a suppressor region in the promoter (-2.1 - -2.5 kb). Therefore, we made constructs harboring the truncated versions of DLK2 promoter and the intron. In case the DLK2 intron is attached to the OP fragment (the resulting construct is OPi; Figure 6), we observed a strong GUS expression in roots, senescent leafs, hypocotyls, cotyledons, stomata guard cells, meristems and flower buds. Interestingly enough, a short (1.2 kb) version of OP (IP; Figure 6) induces the same expression pattern as OPi, but without the intron. In IP plants, various hydroponic treatments with GR24 racemic mixture or (+) and (−) strigol enantiomer pairs, and furthermore, fluence rate greatly determines the pattern of the GUS expression. In case the intron is attached to the IP contruct (IPi), the GUS activation can only be observed in the meristem, the basal region of petioles and flower buds, and GUS expression is highly dependent on the strigolactone type used.

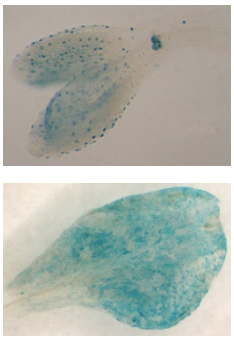

Figure 6: Col-0 plants harboring the IP (above; 8 days old seedling grown on MS plates) or OPi (down; senescent leaf) constructs show distinguished GUS expression in stomata guard cells, meristems and leaf blade.

Taken together the microarray and the GUS assay results, and the publications reporting on the role of strigolactones in light signalling, we assumed that by using various light regimes we could tease out the subtle phenotype of *dlk2* mutants. We applied continuous white, blue, red and far-red fluences as well as four different white light fluence rates on young *dlk2*, *kai2*, *d14*, Col, Ler and DLK2OE (overexpressing) seedlings. We found that *dlk2* seedlings showed a distinct, very large cotyledon phenotype under low fluence rate (10 µmol m^{-2} s^{-1}), while DLK2OE plants showed the opposite, small cotyledon phenotype (Figure 7). We conclude that DLK2 is required for normal cotyledon growth under low light conditions.

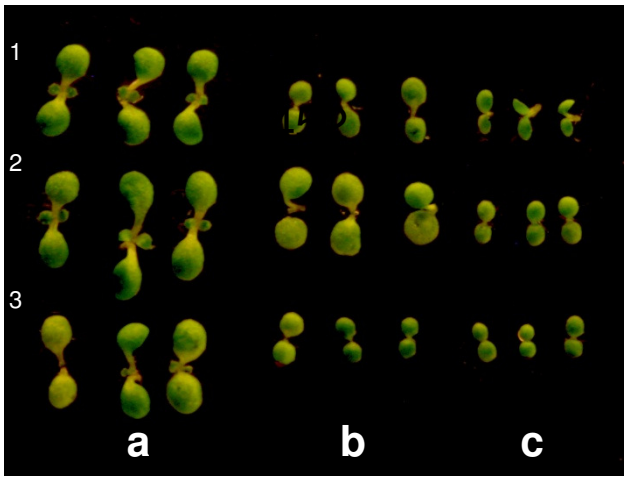

Figure 7: 12 days old *dlk2* seedlings display a large cotyledon phenotype under low fluence rates. 1: Ler; 2: *dlk2*; 3:DLK2OE. a: normal light fluence (100 µmol m^{-2} s^{-1}), b: low light fluence (10 µmol m^{-2} s^{-1}), c: low light fluence (10 µmol m^{-2} s^{-1}) + MS media supplemented with 10 uM GR24.

Genetic screens to identify genes responsible for TMB insensitivity and hypersensitivity

We also employed a genetic screen in lettuce to find out what genes and network might lay behind TMB action. First, 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 these two parental lines has been generated and genotyped previously and is now ready to use. 160 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, 19 cM each (Figure 8). Now we are in a phase of designing further experiments and genotyping to narrow down the list of candidate genes.

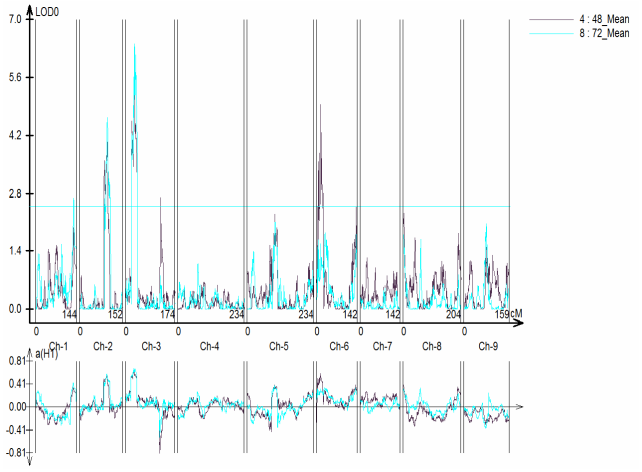

Figure 8: QTL analysis of TMB responsiveness of a *Lactuca sativa* 'Salinas' X *Lactuca serriola* RIL population. Two distinct QTLs on chromosome 2 and 3 have been found, each encompassing 19 cM. QTLs were inherited from the maternal (L. 'Salinas') lines, therefore regarded as a TMB insensitivity trait.

In-depth promoter analysis of the butenolide – responsive candidate genes

We also made attempts to map transcription factor binding sites of the selected KAR1 or TMB – related promoters and to use biotinylated fragments of promoters for streptavidin pull-down assay in order to select transcription factors. We picked DLK2 and FUS3

promoters (this latter gene has been marketedly upregulated after TMB treatment and is related to ABA action). For pilot studies, we chose two 50bp long fragments containing known ABRE motifs in each promoter. The fluorescent EMSA assay showed that both fragments can bind specific binding proteins when treated with nuclear protein extracts. Then, we subjected the fragments labelled with Cy3 to DNase I treatment in order to map binding sites (DNase footprinting). First, we needed to devise a method to generate fluorescent Maxam-Gilbert ladders, as we've found no literature describing fluorescent ladders. With the minor modification of the original method (oxidized piperidin extincts the fluorescence of Cy3 and Cy5), we managed to prepare fluorescent ladders, however, at a small scale. The fluorescent DNase I footprinting assay resulted in a distinct fingerprint of the nuclear extract, however, no difference was found between treatments (control vs KAR1 or TMB). We also made several rounds of trials with biotin-labelled promoter fragments to pull down transcription factors, but the results were inappropriate. Nuclear extracts were treated with the biotinylated fragments, then they were pulled down with magnetic beads, and the extracts were subjected to 2D PAGE. Although we managed to identify proteins, the results were inconsistent with our expectations, as albumins, heat shock proteins and common histones were overwhelmingly represented. We think that the further optimalization of these methods and the adaptation of novel achievements (e.g. ChIP - on - Chip) might worth the efforts in the future, however, the present time frame of this proposal does not allow the extensive optimalization of these labour-intensive methods.

Conclusions

Plants interaction with their environment substantially consist of sensing the environmental cues surrounding the plant and responding to the stimuli. In this project we focused on novel emerging signal molecules bearing a common butenolide moiety. These butenolide compounds, karrikins, TMB and eventually strigolactones, can orchestrate the molecular processes lie behind, from stress responses to plant architecture, germination to seed setting, and therefore, have a huge impact on the sequence of events shaping a plants life. We demonstrated that butenolides can induce contrasting transcriptional events during germination, mainly through light signaling. We found DLK2, the paralogue of strigolactone receptors, as a key player in strigolactone/karrikin signaling, which orchestrates strigolactone-dependent low light fluence responses in young seedlings. Our original aim to gain insight into the subtle events how these compounds work was accomplished, and we open the door for further research zoomed at the role of DLK2 and butenolide derivatives in seed germination, seedling morphology, light signaling and the regulation of plant architecture.

Manuscripts related to the present project:

Pošta M, Soós V and Beier P (2015) Design of photoaffinity labeling probes derived from 3,4,5-trimethylfuran-2(5*H*)-one for mode of action elucidation. Manuscript submitted to Molecules.

Soós V, Végh A, Posta M, Kátay Gy, Czvaczka J, Huo H, Bradford K, Balázs E (2015) The D14 paralogue DLK2 regulates strigolactone – dependent low fluence responses in Arabidopsis seedlings. Manuscript under preparation (to be submitted to Plant Physiology).

Soós V, Végh A, Posta M, Yoong FY, Niroula M, Huo H, Bradford K, Balázs E (2015) QTLs responsible for TMB insensitivity in *Lactuca sativa* 'Salinas' X *Lactuca serriola* RIL population. Manuscript under preparation (to be submitted to Plant Physiology).

Ghebrehiwot H M, Kulkarni MG, Szalai G, Soós V, Balázs E, Van Staden J (2013). Karrikinolide residues in grassland soils following fire: Implications on germination activity. South African Journal of Botany, 88, 419-424.

Soós V, Sebestyén E, Posta M, Light ME, Kohout L, Van Staden J and Balázs E (2012) Molecular aspects of the antagonistic interaction of smoke-derived butenolides on the germination process of Grand Rapids lettuce achenes. New Phytologist 196(4):1060-73