Characterization of the strigolactone biosynthesis-related *D27-LIKE1* gene in plants (FK128637)

Final report

Summary

The main objective of the proposal is the characterization of the *D27-LIKE1* gene (which encoding a presumed beta-carotene isomerase) in plants. During our experiments, we primarily focused on the Arabidopsis *D27-LIKE1* (*AtD27-LIKE1*), but we also conducted experiments with the barley orthologue (*HvD27-LIKE1*). Our research plan was based on our earlier work in which we examined H_2O_2-sensitive miRNAs and their target genes in wheat. During our research, we characterized the *D27-LIKE1* gene and identified its physiological and enzymatic function in Arabidopsis. The *d27-like1-1* mutant did not show strigolactone (SL) deficiency traits and has a significantly higher 9-*cis*-violaxanthin content, which is associated with a slightly higher abscisic acid (ABA) level. In *in vitro* feeding experiments, the recombinant D27-LIKE1 protein showed affinity for all beta-carotene isoforms, but more efficiently participates in the transformation between *trans/cis* and 9-*cis*, 13-*cis*, 15-*cis*-β-carotene forms. Additionally, it also accepts zeaxanthin and violaxanthin as substrates. We confirmed that *D27-LIKE1* mRNA is mobile in phloem and that D27-LIKE1 is an ancient isomerase with a long evolutionary history. In summary, we have proven that D27-LIKE1 is a carotenoid isomerase that accepts various carotene and xanthophyll isoforms as substrates, but its primary role is in *cis/cis* transformations. Therefore, D27-LIKE1 could potentially regulate the *cis*-carotenoid pools and, consequently, the SL and ABA biosynthesis pathways. Through our experiments with barley, we managed to produce valuable genetic material that can be used in future research. We published our results in a D1 internationally recognized journal and also presented them at an international conference. We consented that the internationally known TAIR Arabidopsis database (https://www.arabidopsis.org/) attaches the described function and the published article to the gene's datasheet.

Arabidopsis studies

Ancestry of the DWARF27 family

Our target gene, *D27-LIKE1* is a member of the D27 family, which consists of three paralogs with a long history of evolutionary divergence. The D27 (DWARF27) catalyzes the first step in strigolactone (SL) biosynthesis converting all-*trans* beta-carotene to its 9-*cis* isomeric form. The other two paralogs, D27-LIKE1 and D27-LIKE2 have no assigned function yet. At first, we made a widespread phylogenetic analysis using protein sequences of D27-LIKE accessions from numerous algae and land plant species (ONEKP and NCBI databases). Based on our analysis, D27 family consists of four distinct clades, D27-LIKE3, D27-LIKE2, D27-LIKE1 and D27 (*Gulyás et al. 2022, Plant Journal*). Interestingly, the fourth member of the DWARF27 family (D27-LIKE3) is completely absent in seed plants and gymnosperms unexpectedly lost D27, suggesting that other paralogs might replace its functions.

Phenotyping the *d27-like1-1* mutant

To functionally characterise D27-LIKE1, we used insertion mutant line (GT13552) of AtD27-LIKE1 (D27-LIKE1) from the L*er* genetrap collection (Cold Spring Harbor Laboratory). In GT13552, insertion is located within the first exon of *D27-LIKE1*. After backcrossing three times, a line homozygous for GT13552 allele was obtained and the progeny of this line was termed as *d27-like1-1*. Following this, we explored the phenotypic attributes associated with the previously mentioned paralogue, AtD27 (D27). The *d27* mutants of Arabidopsis exhibit a more branched appearance and are slightly shorter in stature than their wild-type counterparts. However, such characteristics were not evident in the *d27-like1-1* mutant (Fig. 1a). When comparing *d27-like1-1* and L*er* plants, we detected no variations in leaf size or area (Fig. 1b). This suggests that D27-LIKE1 is not a primary contributor to SL synthesis. Subsequently, we explored if the karrikin signalling, facilitated by KAI2 (a paralogue of D14), is altered in d27-like1-1 plants. Notably, there was no discernible difference in root hair density between wild-type and *d27-like1-1* plants (Fig. 1d). This, along with the hypocotyl observations, hints that the karrikin signalling pathway remains intact in *d27-like1-1* plants (Fig. 1c).

Studies related to localization, promoter activity and mobility of D27-LIKE1

During the proposal, we determined the subcellular localization of the D27-LIKE1 protein with transient expression assay in protoplast (35S:cD27-LIKE1:GFP**)**. Similarly to the paralog D27, D27-LIKE1 is localized in plastids. To understand spatio-temporal dynamics of the *D27-LIKE1* promoter activity, we examined GUS expression across three developmental stages. In 7-day-old seedlings, robust activity was seen in the cotyledon, with inconsistent expression in the hypocotyl. Both primary and lateral root tips exhibited GUS signals in the elongation zone. In 4-week-old rosettes, strong activity was observed in the petiole and midrib, but this diminished towards the leaf edge. By the reproductive stage, stems showed no signal, while roots retained a similar expression pattern as young seedlings in the elongation zone.

Notably, there was pronounced GUS staining in the meristematic zone. Within flowers, the stigma had high GUS activity, while sepals and stamens had minimal expression.

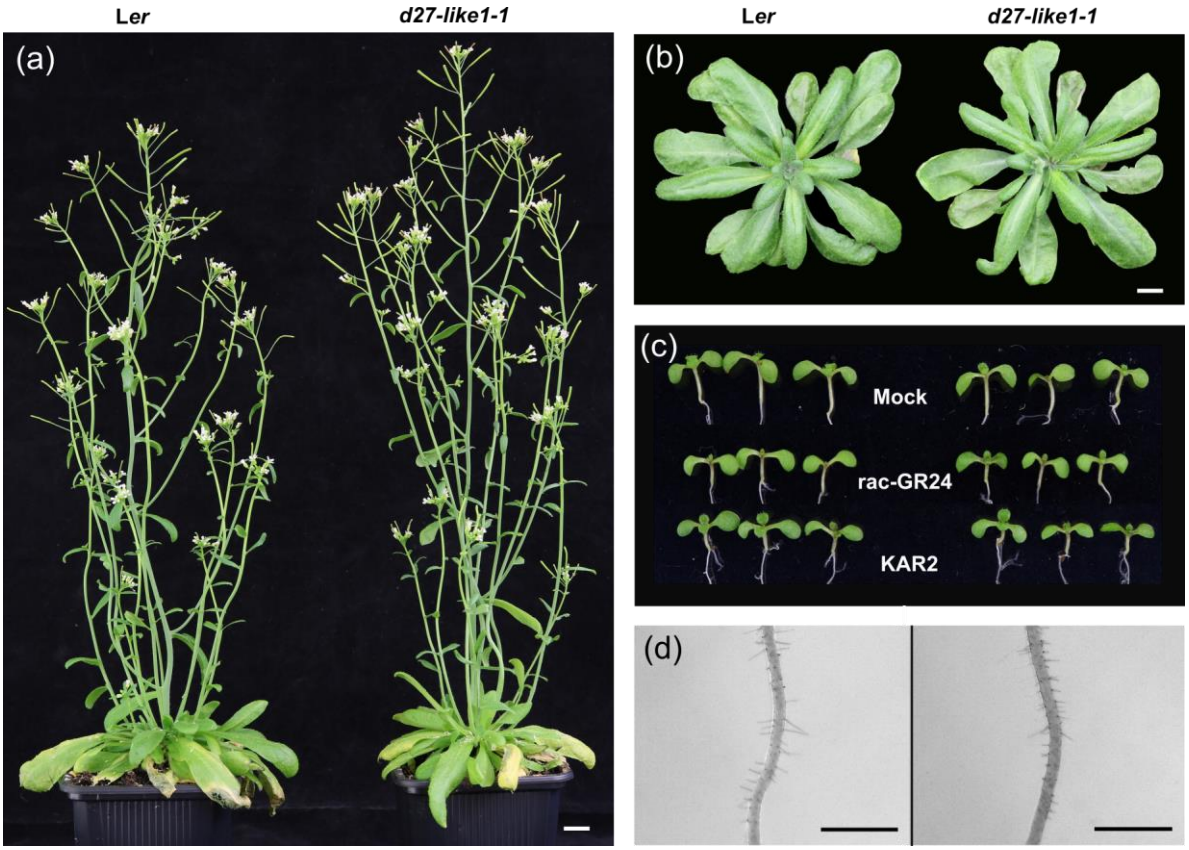

Figure 1. D27-LIKE1 does not have a prominent role in SL or KAI2 ligand biosynthesis in Arabidopsis. (a) Phenotype of wild-type Ler (left) and d27-like1-1 (right) adult plants. The plants shown are 6 weeks old and were grown under short-day (8:16 h) conditions for 4 weeks and then under long-day (16:8 h) conditions for 2 weeks. Scale bar = 1 cm. (b) 5-week-old rosettes of wild-type Ler (left) and d27-like1-1 (right) plants. Plants were grown under short-day (8:16 h) conditions. Scale bar = 1 cm. (c) Hypocotyl elongation of 10-day-old seedlings (Ler left, d27-like1-1 right) after 1 μM rac-GR24 and 1 μM KAR2 treatments. Scale bar = 1 mm. (d) Root hair density of 10-day-old seedlings (Ler left, d27-like1-1 right). Scale bars = 1 mm.

To detect the presence of *D27-LIKE1* mRNA in the phloem, we utilized the droplet digital PCR device available at our institute. We confirmed that *D27-LIKE1* mRNA is present in the phloem and is transported from the shoot to the root (Fig. 2a). In conjunction with this, we grafted L*er* scions onto *d27-like1-1* rootstocks. Two weeks post-grafting, we identified *D27-LIKE1* mRNA in the *d27-like1-1* roots, verifying our earlier findings (Fig. 2b).

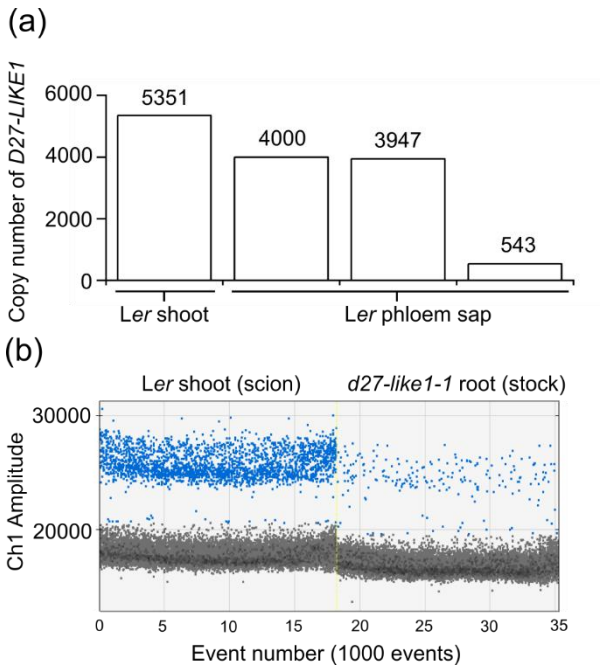

Figure 2. *D27-LIKE1* mRNA is phloem mobile and transported from shoot to root (a). Copy numbers were normalised and are expressed as per 1000 copy numbers of *UBQ10*. One-dimensional plot of ddPCR assay showing the presence of *D27-LIKE1* mRNA in the roots of a L*er*|*d27-like1-1* grafted seedling (b). Positive droplets, containing the target DNA are shown with blue and negatives with grey colours. The *x*-axis represents the total number of droplet events, The *y*-axis represents the detected fluorescence amplitude. Experiments were repeated in biological triplicates (n = 3).

Carotenoid profiles in *d27-like1-1* mutant, *D27-LIKE1* overexpressing lines and in complemented *d27-like1-1* lines

As our target gene, *D27-LIKE1*, was predicted to be a putative beta-carotene isomerase, we investigated the carotenoid profiles in our mutant line and complemented lines. In our experiments, numerous carotenoids (12) were measured across two different laboratories: the Agricultural Institute in Martonvásár (conducted by Kamirán Áron Hamow) and the Biological Research Centre in Szeged (conducted by László Kovács), using two different analytical systems. We found two differences in carotenoid profiles between *d27-like1-1* and wild-type plants. In the mutant plants, the level of 9-*cis*-violaxanthin was threefold higher than in the L*er* (Fig. 3a). Meanwhile, 9-*cis*-β-carotene showed a modest rise (20%) in the mutants (Fig. 3b). In parallel with this experiment, we examined the carotenoid profiles in *D27-LIKE1* overexpressing lines. However, we found no differences in carotenoid levels when compared to the wild-type. Our complemented mutant lines displayed a profile similar to the wild type, further confirming the relationship between the mutation and the increased 9-*cis*-violaxanthin level (Fig. 3).

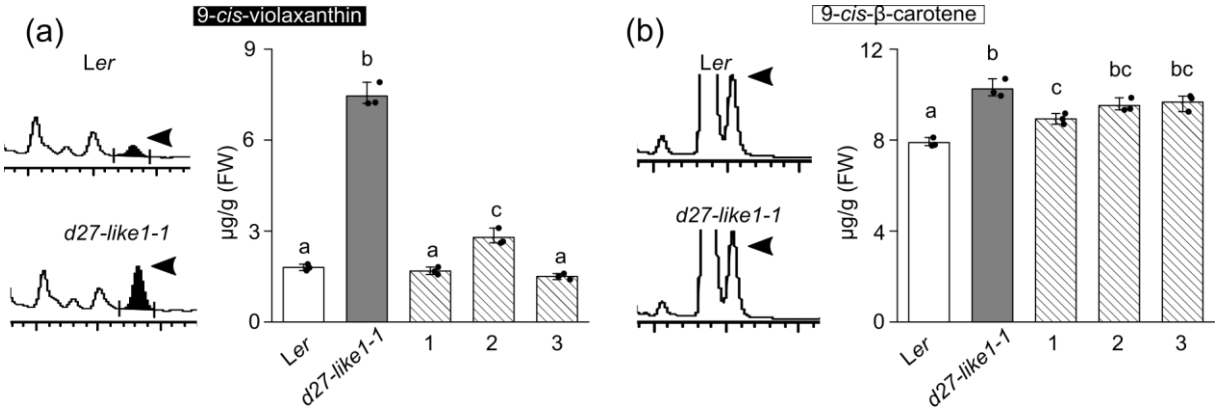

Figure 3. The rosettes of *d27-like1-1* mutant accumulate 9-*cis*-violaxanthin. Enlarged HPLC chromatograms of 9-*cis*-violaxanthin and 9-*cis*-β-carotene peaks. 9-*cis*-violaxanthin (a) and 9-*cis*-β-carotene (b) content of 2-week-old L*er, d27-like1-1* and three complemented mutant lines (denoted as 1, 2 and 3). Bars with the same letter are not significantly different from each other (n = 5, 5 rosettes per genotype in each; mean ± SD; anova, $P < 0.05$, Tukey's test).

Recombinant D27-LIKE1 mainly catalyses *trans/cis, cis/cis* isomerisation of carotenoids in vitro

The purified recombinant D27 and D27-LIKE1 proteins were incubated with different isoforms of carotenes and xanthophylls. Our investigated protein, D27-LIKE1 can convert the all-*trans* β-carotene into different *cis*-β-carotene forms (9, 13, 15). D27 protein showed higher affinity towards *trans*-β-carotene, as previously described. When our proteins were fed with *cis*-β-carotene forms, D27-LIKE1 had significant activity in the interconversion among these *cis*-β-carotene forms (Fig. 4a). We can conclude that D27-LIKE1 is a preferential *trans/cis* and *cis/cis* isomerase, whereas D27 is more capable of catalysing the reversible conversion between all-*trans*-β-carotene and 9-*cis*-β-carotene.

In a separate experiment, we incubated recombinant proteins with different zeaxanthin and violaxanthin isoforms. Both D27 and D27-LIKE1 were able to convert all-*trans*-violaxanthin into 9- and 13-*cis*-violaxanthin. However, when supplied with a mixture of trans-*cis*-violaxanthin, there was a notable decrease in the 9-*cis* form and a minor increase in the 13-*cis* form in the presence of D27-LIKE1 (Fig. 4b). This result underscores the crucial role of D27-LIKE1 in *cis-cis* interconversion. Compared to the control, both recombinant proteins were capable of converting all-*trans*-zeaxanthin to its *cis* isoforms, but D27 proved to be more efficient. The assay demonstrated a significant decrease in 9-*cis*-zeaxanthin and a minor increase in 13-*cis* isomeric forms with D27-LIKE1, paralleling the results observed with the *trans-cis*-violaxanthin mixture.

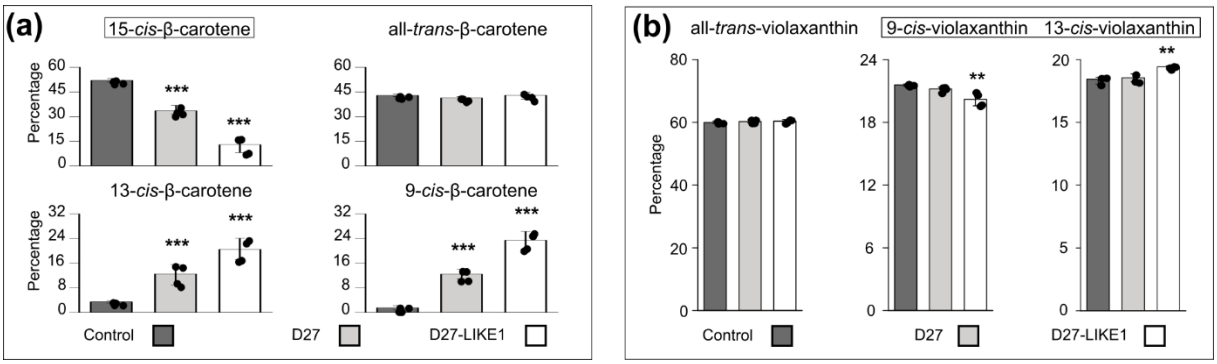

Figure 4. *In vitro* feeding experiments were performed with recombinant D27-LIKE1, D27 and void construct (control) with various substrates. Percentage of different *cis*- and *trans* isomers (boxed names) when (a) 15-*cis*-ß-carotene, (b) mixture of *cis*-violaxanthin isomers (9-*cis*, 13-*cis*) were incubated with 50 µg of recombinant D27 or D27-LIKE1 protein. Data are the means of four biological replicate experiments in each (n = 4). Bars with asterisks are significantly different from the control (mean ± SD; Anova, *P < 0.05, **P < 0.01, ***P < 0.001, Tukey's test).

d27-like1-1 mutant displays a moderate increase in ABA and phaseic acid content

d27-like1-1 plants shows remarkable 9-*cis*-violaxanthin accumulation, which might eventually result in a higher ABA content. We measured ABA, phaseic acid (PA) and dihydro-PA levels in 14-day-old rosettes and freshly harvested seeds of *d27-like1-1* and L*er* plants. ABA and its catabolites displayed an approximately 1.6–1.9-fold increase in *d27-like1-1* in both rosettes (Fig.5a.) and seeds. We found that this slightly elevated constitutive ABA level of *d27-like1-1* plants is not necessarily manifested in higher stress tolerance, though the salinity tolerance of germinating *d27-like1-1* seeds was definitely better than wild-type.

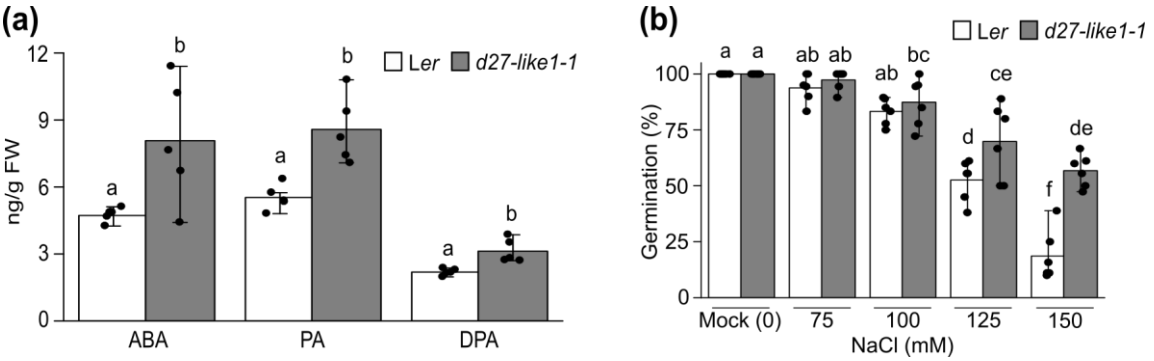

Figure 5. *d27-like1-1* mutant exhibits a moderate increase in ABA content. ABA, PA and DPA levels, as well as the salt stress and dehydration tolerance, of *d27-like1-1* was investigated. (a) ABA, PA and DPA content of 2-week-old detached, non-stressed rosettes of L*er* and *d27-like1-1* plants, respectively. (b) Germination efficiency of *d27-like1-1* on NaCl supplemented plates. Germination percentages were assessed in 7-day-old seedlings. Data are the means of three independent biological replicate experiments, with > 60 seedlings per genotype in each.

Conclusion

Based on our results, we suggest that, in Arabidopsis, D27-LIKE1 mainly functions as a violaxanthin isomerase in plants, ensuring a balance between *trans* and *cis* isomers. This leads to a heightened presence of 9-*cis*-violaxanthin in mutant plants. The subsequent increase in ABA in *d27-like1-1* and the lab-based tests, where D27-LIKE1 drove the *trans/cis* and *cis/cis* changes in violaxanthin, lend strong support to this theory. Nevertheless, it plays an important role in the interconversion of *cis*-β-carotene forms, regulating the cis pools. Through this function, it can influence SL biosynthesis in conjunction with the dominant player, D27.

Barley studies

We planned to create *HvD27-LIKE* mutant lines using the CRISPR/Cas9 genome editing technique with the pHUE411 binary CRISPR vector (www.addgene.org). In the third year, we performed another barley transformation, during which we managed to select a line that contained our CRISPR construct. After inbreeding, we examined several plants from this line in the fourth year to see if the desired mutation had occurred in our targeted gene. Although the plants contain our constructs, unfortunately, we did not find a mutant plant. Due to increased energy prices, we do not have enough resources from the grant to initiate another transformation material, so we tried to validate the function of the *HvD27-LIKE* gene with simpler experiments in the final year of the project.

One approach was to complement our Arabidopsis *d27-like1-1* mutant with the barley *HvD27-LIKE* gene. Currently, about 10 lines have been selected and inbred. The homozygous lines are available to us, so based on the knowledge from our Arabidopsis studies; we will have opportunity to characterize the barley D27-LIKE1 in the future projects.

One of the strengths of the results is that we fed *AtD27* and *AtD27-LIKE1* recombinant proteins with various carotenoids (*cis* and *trans* isomers) during "feeding" experiments. Then, we examined which carotenoid showed activity with our recombinant proteins. The production of the barley HvD27-LIKE recombinant protein is also underway. However, we could not finish our experiments in this grant.

Justification for deviations from the plan

The work related to Arabidopsis *D27-LIKE1* was fully completed as planned. No publication has resulted from the research in barley, but valuable genetic material has been produced, which may be promising for our upcoming experiments.

The reasons:

Due to the effects of the COVID-19 pandemic in 2019-2020, the execution of certain work processes was hindered (working from home). In the final year of the grant (2023), the increased energy prices meant that the institute's phytotrons could not be used. Nevertheless, three colleagues left the institute, who had important roles in the grant. Unfortunately, we could not replace them with a suitable workforce. In light of the above, please accept my publications,

which I made in the home office. One of these publications is a review titled "*Light-dependent regulatory interactions between the redox system and miRNAs and their biochemical and physiological effects in plants.*" This topic is indirectly related to this grant, as we first encountered *D27-LIKE1* in our previous miRNA study (Cao et al. 2019). The other publication is a short article titled "*α-Aminoadipic acid metabolism is controlled by the glutathione-dependent redox environment in Arabidopsis*" which was written in the home office based on our previous experiments.

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