Final report

The central hypothesis of our research project was that elevated ambient temperatures are linked to plant morphogenesis in two ways: i) although the plant structure is altered primarily via cell elongation to ensure better adaptation ii) the increased cell division rate of the meristems also contributes to the accelerated development at higher temperatures. Based on our unpublished preliminary data we hypothesized that: i) the receptor-like cytoplasmic kinase RLCK VI_A2 has a potential role in thermomorphogenesis (TM) to fine tune cell elongation ii) the RBR-E2F regulatory module is important to maintain coordinated cell division and differentiation under elevated temperatures. We also planned experiments to study how much the ambient temperature responses of Arabidopsis (dicot) and Brachypodium (monocot) are similar or different. Here, we outline the experimental methodologies employed and the outcomes obtained in response to the hypotheses put forth in the project proposal.

1. The function of the RLCKVI_A2 kinase

Considering the potential involvement of the RLCK VI_A2 kinase we had two tasks: i) phenotypic analysis of plants with altered RLCK VI_A2 levels at different ambient temperatures ii) and the identification potential downstream targets and upstream regulators.

1.1. Phenotypic analysis of the thermomorphogenic response

Thermomorphogenic screening was conducted with the rlck vi_a2 mutant, along with other available mutant/transgenic lines related to RLCK VI_A2 signalling (*35S:RLCK VI_A2; brm1, rpl_4, rop11, rop3, rop2_1, 35S:ROP2CA, rop2_1 35S:ROP2CA, rop2_1 35S:ROP2*) in plant culture cabinets. The screenings first took place under *in vitro* conditions, at 22 and 28 °C, with short and long day, across three independent replications. The results were inconclusive and did not agree with the preliminary data. It could partly be associated with the uneven in vitro conditions from experiment to experiment. We also proposed the analysis of the plants thermomorphogenic response in a newly established plant phenotyping system. Unfortunately, the setting up of the system was considerably delayed, in part due to the COVID pandemic, in part because the system was a prototype. We were only able to carry out the experiments in the second year, growing one-week-old plants (20 per line) for two weeks at 22 or 28 °C under greenhouse conditions. Then, a new problem arose because the person trained to analyse the experimental data produced by the system left the institute. It took another year to have someone to extract the raw data out of the automatically taken images. Until now, only the preliminary analysis of the huge amount of data became possible in the absence of the required expertise. Nevertheless, we could obtain useful information even from the preliminary data. Plant size, growth rate, and compactness (leaf coverage) parameters were determined for two *rlckvi_a2* mutants, and mutants in the genes coding for associated proteins such as BRM (potential substrate), RPL (potential upstream regulator), ROP2 and ROP11 (interacting regulatory partners). The obtained data could not confirm the significant involvement of the RLCKVI_A2 kinase or its associated signalling pathway in the thermomorphogenic response. On Figure 1, comparison of the growth and morphology parameters of Col-0 wild type and *rlckvi_a2* mutant (gk18) plants are shown as example (gk18 is the abbreviation of the accession number of the mutant).

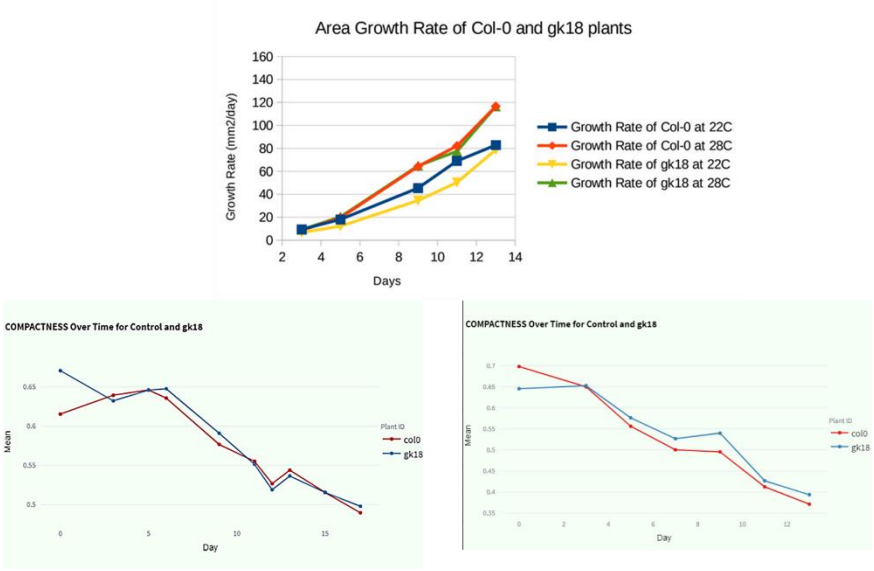

Figure 1. The growth rate and compactness (an estimate of leaf coverage) parameters of Col-0 wild type and rlckvi_a2 mutant plants growing at 22 or 28 °C are based on images automatically taken by the phenotyping system. Each datapoint represents averaged data of twenty individuals.

In summary, despite our expectations, the phenotypic analyses did not confirm the involvement of the RLCKVI_A2 kinase in thermomorphogenesis and contradicted the preliminary experiments. However, since we could unambiguously draw this conclusion only in the third year, we carried out in parallel the proposed experiments to functionally link the kinase to its potential upstream regulators and downstream targets.

1.2. Identification of RLCK VI_A2 partners and potential upstream and downstream events

1.2.1. Downstream targets

The BRM protein was reported to be potentially involved in thermomorphogenesis (Torres and Deal, 2019). We have previously found that a partial BRM polypeptide interacts with the RLCKVI_A2 kinase in yeast two-hybrid assay, the RLCKVI_A2 can in vitro phosphorylate the BRM protein and might target it for degradation (A. Fehér: ROP GTPase-activated kinase signalling in Arabidopsis; Plant Biology Europe, Torino, Italy, 2020, 28.06.-01.07. 2021). Since the above experiments were based on ectopic expression of the proteins, there was a need for in vivo proof of the functional interaction between the proteins in order to publish the results. To help in planta analysis of RLVK VI_A2 BRM functional interaction, anti-BRM antibody was ordered from a specialised company. Unfortunately, the produced antibody could not recognise the BRM protein in plant cell or nuclear extracts. As a potential way to verify in planta RLCKVI_A2 BRM interaction, as well as to identify further potential substrates of the kinase, the RLCKVI_A2 kinase was expressed in Arabidopsis seedlings fused to the HA:TurboID tag. This tag allows biotin proximity ligation to interacting proteins. Biotinylated proteins were identified by streptavidin-affinity purification followed by mass spectroscopy in the central proteomic laboratory of the host institution. The list almost exclusively contained protein kinases indicating that RLCKVI_A2 might be involved in several kinase signalling pathways. The validation of such interactions is rather difficult (which kinase phosphorylates which and how it affects the activity of the target towards unknown substrates). Another difficulty arose when the commercialization of radioactive isotopes used for in vitro kinase

assays ended in Hungary. Furthermore, we could not identify any overlap among the protein lists obtained by phosphoproteomic comparison of the rlckvi_a2 kinase to wild type, by yeast two hybrid screening of Arabidopsis cDNA libraries using RLCKVI_A" as bait, co-immunprecipitation of GFP-tagged RLCKVI_A2 together with interacting proteins (previous project: K 124828) and the use of the TurboID tag for in planta kinase partners (this project). Therefore, the identification of downstream RLCKVI_A2 substrates was not anymore, a reasonable pursuit.

1.2.2. Upstream regulators

We have previously established (project: K 124828) that the expression of the RLCKVI_A2 kinase is controlled by the REPLUMLESS (abbreviation RPL, other name BELLRINGER) transcription factor, which is a central regulator of shoot development. We have expressed the RLCKVI_A2 kinase under the control of a constitutive promoter in the wild-type, the *rlckvi_a2*, and *rpl-4* loss-of-function mutant backgrounds. We continued the detailed characterization of these lines and established that although the RLCKVI_A2 and RPL proteins are involved in similar processes such as replum development, phyllotaxis, stem growth and vascular organisation, the ectopic expression of the kinase cannot complement either the *rlckvi_a2*, or the *rpl-4* loss-of-function mutations. Instead, it has a dominant phenotype increasing stem diameter and the number of vascular bundles per stem (Fig. 2).

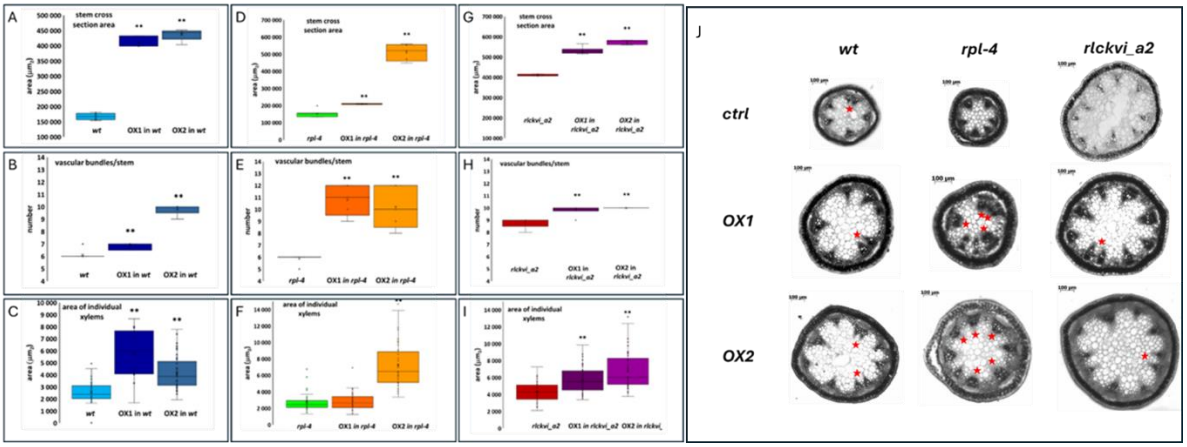

Fig. 2. Effect of ectopic RLCKVI_A2 expression on stem thickness and the vasculature. A-I: Stem cross section area (A, D, G) the number of vascular bundles per stem (B, E, H), and the average size of xylems (C, F, I) were determined for wild-type (wt; A-C), rpl-4 (D-F), or rlckvi_a2 (G-I) plants without and with overexpression (OX1 and OX2) of the 35S:RLCKVI_A2 gene. Ten plants per line were measured. The distribution of the measured values is represented as a box plot for each line. The data of the overexpressor lines (OX1 and OX2) were compared to their respective control using Student's t-test ($p < 0.05$ = *; $p < 0.01$ =**). J: Stem cross sections of wild-type (wt) and mutant plants (rpl-4 or rlckvi_a2) without and with overexpression (OX1 and OX2) of the 35S:RLCKVI_A2 gene. Red asterisks indicate closely placed/clustered vascular bundles.

These results have recently been submitted for publication for the journal IJMS (IF 5.6, Q1 in "Biochemistry & Molecular Biology"; Special Issue "Modern Plant Cell Biotechnology: From Genes to Structure 2.0"). The manuscript can be seen as a preprint at preprint.org with the id: preprints-111276.

2. The involvement of the RBR-E2F regulatory module in the thermomorphogenic response

Ambient temperatures have a major effect on plant growth and development, and in turn, contribute to triggering the response of the plant to seasonal and climatic changes. The rate of cell division in the meristems and the elongation of differentiating cells determine the rates of plant and organ growth. Despite this fact, our knowledge about the temperature regulation of meristem function is rather limited. Although plant thermomorphogenesis has recently attracted considerable research interest due to the forecasted climate change, it has mainly been focused on temperature sensing and signalling pathways resulting in hypocotyl elongation (Quint *et al.*, 2023). This has revealed many molecular details of the process (Wu *et al.*, 2023). However, the thermomorphogenic response of the root and the effect of elevated ambient temperatures on cell divisions in the meristems have been neglected until recently. It was only after we had initiated our project that the effects of temperature on auxin homeostasis in the root and cell divisions in the root meristem were first published (Ai *et al.*, 2023). This study supported our hypothesis that temperature influences meristem function, but it could not provide a link between temperature-regulated auxin and cell division control. As we proposed in our grant application, we focused our research on the RBR-E2F regulatory hub central to control plant cell division and differentiation in the meristems.
Since the most important results are already available online as a preprint (https://www.biorxiv.org/content/10.1101/2024.05.28.596227v1), here we focus on a general description including some of the yet unpublished details.

2.1. Phenotypic analysis of the thermomorphogenic response

According to our proposal, we studied hypocotyl and root growth of various Arabidopsis E2F (E2FA, E2FB, E2FC) and RBR mutants at two different temperatures, at 22^{o}C and 28^{o}C compared to the wild-type control. Phenotyping was made on *in vitro* grown seedlings and on mature plants grown in the phenotyping system.
An intriguing observation was made regarding the contrasting effects of the cell cycle activator E2FB and the cell cycle repressor E2FC on the hypocotyl growth of seedlings. Mutations in E2FB (e2fb-1, e2fb-2) led to increased while that of E2FC (e2fc-1, e2fc-2) reduced hypocotyl length compared to the wild type at 22 °C. The hypocotyls of these mutants, however, could not significantly elongate more at 28°C than at 22°C, unlike those of the control. The E2FA mutants (e2fa-1 and e2fa-2) were more like those of E2FB, while the triple e2fabc mutant was more similar to those of E2FC. Furthermore, the double mutants combining E2FC mutations with those of E2FA or E2FB had reduced hypocotyl length like the e2fc-1 single mutant but most of them exhibited the thermogenic response. These observations suggest the contrasting involvement of activator and repressor E2Fs in hypocotyl growth at 28°C. This was the first observation that core cell cycle regulators can influence hypocotyl (cell) elongation.
The response of Arabidopsis seedlings with different RBR levels (wild type Col-0, mutant rbr1-2, and the overexpressor expressing pRBR1::gRBR1GFP designated further as RBR::GFP) to elevated temperatures was also surprising. While the response of the rbr1-2 mutant was somewhat less strong than that of the wild type, the RBR::GFP line exhibited a huge enhancement in thermoresponsive hypocotyl elongation (Fig. 4). With this line, we contributed to a paper (Lang *et al.*, 2021).

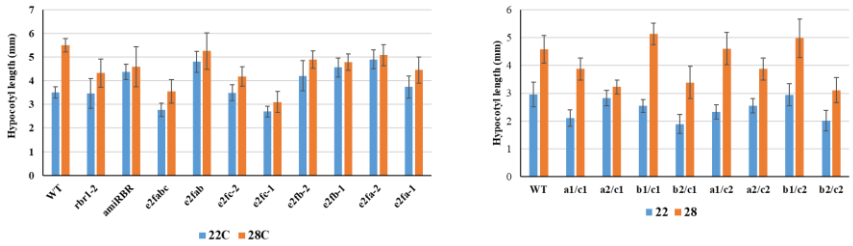

Fig. 3. Effect of various E2F mutant combinations on hypocotyl elongation and its temperature response. Seedlings were grown on the indicated temperatures for 5days. In the e2fabc triple these e2fab mutants were combined: e2fa-1/e2fb-2/e2fc-1. Ten seedlings were used for the hypocotyl measurements. Averages and standard deviations are shown for twenty seedlings.

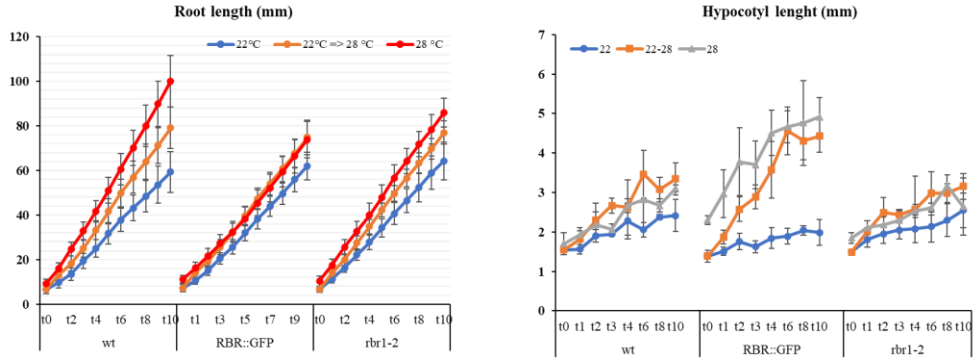

Fig. 4. RBR level affects thermomorphogenic root and hypocotyl elongation. Seedlings were continuously grown at 22 or 28 ºC or transferred after 4 days from 22 to 28 ºC (22-28). t0 is the fourth day after germination (the time when the seedlings were transferred), the growth was followed for additional ten days. Averages and standard deviations are shown for twenty seedlings.

The RBR mutant/overexpressor was also subjected to phenotyping in a growth chamber. Here, one-week-old seedlings were transferred into soil and grown for two weeks at 22 or 28 ºC measuring their growth rate. Although the data analysis could not be fully completed due to above mentioned reasons, it could be established that the mutant and the overexpressor exhibit a contrasting response to elevated temperatures: the temperature responsive growth was inhibited by the rbr1-2 mutation while enhanced by RBR::GFP overexpression (Fig. 5.).

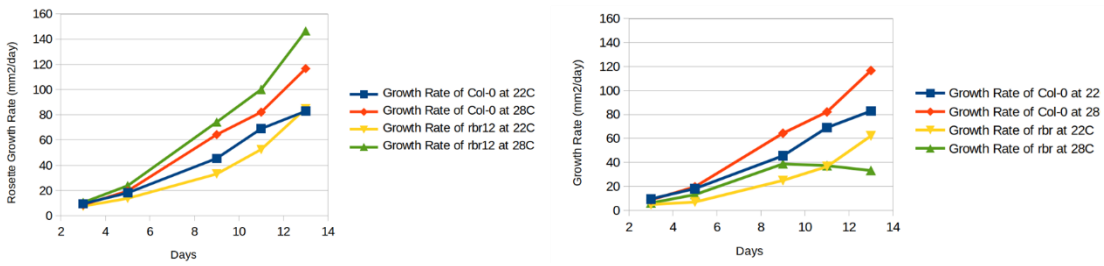

Fig. 5. RBR level affects temperature enhanced plant growth. Seedlings were grown in vitro at 22ºC than transferred to soil and the growth chambers of the phenotyping system. Plant growth was monitored by regularly taking automatic images. Each point represents the averaged data of twenty individuals.

We considered these experiments as convincing evidence of RBR involvement in thermoresponsive plant growth control. Although our preliminary experiments also indicated the involvement of the E2F transcription factors, we decided to focus our further investigations on RBR because: i) the E2F mutants are numerous and difficult to handle, ii) in their case there are no available overexpressors, iii) they are regulated by RBR and therefore the results obtained by RBR might be extrapolated to the RBR::E2F regulatory module.

RBR is primarily considered a negative regulator of cell division and a promoter of cell differentiation. We asked the following questions: i) does meristematic activity is enhanced at elevated temperatures contributing to thermomorphogenesis, ii) and if yes, whether RBR can mediate this temperature control on meristem activity.

The scientific literature contains only limited and contrasting data about the effect of temperature on meristem and especially the effect of temperature on the shoot meristem is neglected functions (Yang *et al.*, 2017; Casal and Balasubramanian, 2019; Ai *et al.*, 2023) . We carried out a detailed analysis of root and shoot meristems following the transfer of seedlings from 22 to 28°C and obtained convincing data that elevating the temperature swiftly increase the activity and size of both apical meristems (Fig. 6).

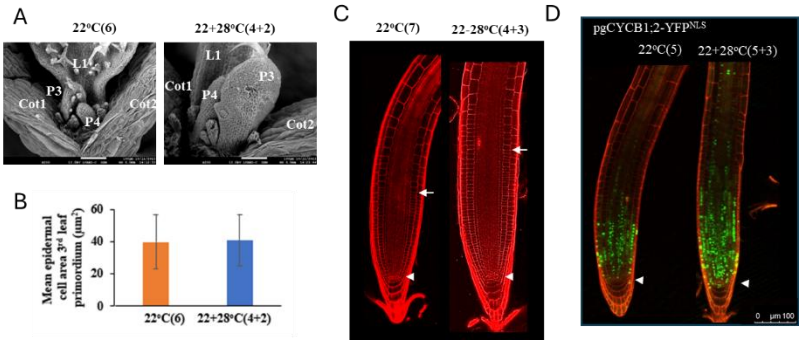

Fig. 7. Effect of elevated temperature on meristem activity. Four- or five-day-old seedlings were transferred from 22 to 28 °C for two or three days, respectively, as indicated. A) Leaf primordia develop larger at 28 °C than 22 °C (compare the size e.g. the third primordia (P3)). B) Primordia are larger at 28 °C, but their cells not, indicating that the larger primordium size is due to more cell divisions. C) The root meristem is longer at 28 °C than 22 °C. D) The cell cycle marker CyclinB::GFP protein accumulates in more cells of the root meristem at 28 °C than 22 °C.

2.2. Involvement of RBR in the thermomorphogenic response

These phenotypic observations were followed by studies that aimed to reveal the involvement of RBR in temperature enhanced meristem activity and hypocotyl elongation.

2.2.1. Regulation of cell division

Based on our results, we suggest that warm temperatures accelerate meristem functions through the conserved CYCD- RBR-E2F molecular pathway. This is supported by the following evidence: (i) the expression of G1 cyclins such as CYCD3;1 and CYCA3;1 was rapidly increases at elevated temperatures (ii) at the same time, the phosphorylation of the cell cycle inhibitor RBR at a conserved CDK site was augmented, and (iii) the expressions of E2F-target

cell cycle regulatory genes ORC2 and CDKB1;1 were also up-regulated with warm temperatures. Furthermore, all of these changes were influenced by the RBR level (Fig. 8.).

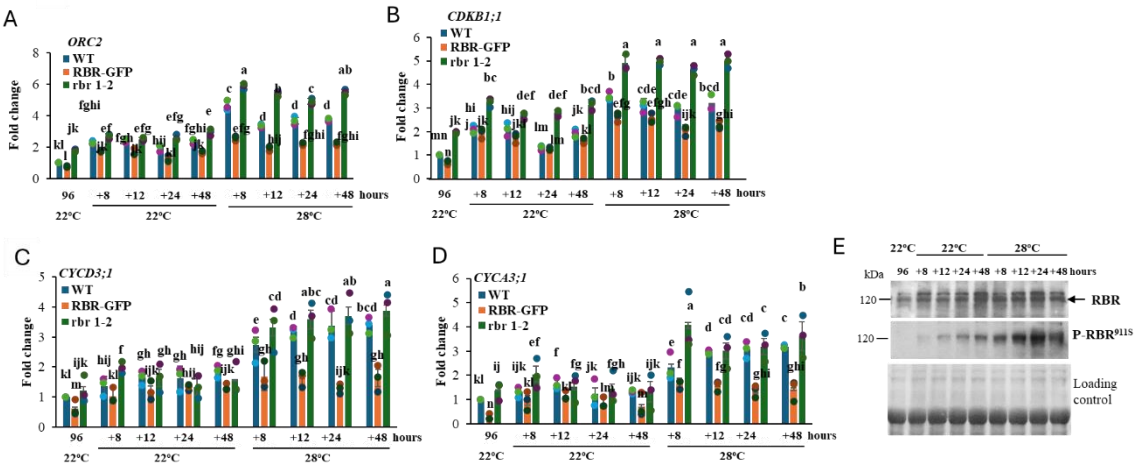

Fig. 7. Warm temperatures suppress the cell proliferation inhibitor function of RBR through activating G1 cyclins and RBR-phosphorylation. A-B) The S- and the G2-M-phase specific ORC2 and CDKB1;1 genes were induced by warm temperatures, depending on RBR. C-D) Transcript levels of the G1 cyclins, CYCD3;1 and CYCA3;1, were elevated by warm temperatures, and are oppositely regulated in the RBR-GFP and rbr1-2 lines. Expression of all the four genes (A-D) was monitored by qRT-PCR. Seedlings of WT, RBR-GFP and rbr1-2 lines were grown for 96 hours at 22ºC after germination and the samples were collected at the indicated time points at 22ºC or 28ºC, respectively. Values represent fold change normalized to the value of the relevant transcript of the seedlings at T0 (96 hours), which was set arbitrarily at 1. Data are means +/- sd. N=3 biological replicates. Different letters mean statistically significant differences ($p < 0.05$) based on one-way ANOVA analyses with Tukey's HSD post-hoc test. E) Western blot shows RBR and phospho-RBR (P-RBR at the 911 serine site) levels in WT seedlings grown as indicated. Sampling was made as in A-D. The membrane was stained with Coomassie brilliant blue to indicate equal loading. Molecular weight marker (kDa) is shown at the left side. Arrow marks the position of the RBR protein.

2.2.1. Regulation of hypocotyl elongation

Unexpectedly, we found that RBR not only functions as a cell cycle inhibitor, but also exerts a positive influence on the elongation growth of post-mitotic, quiescent cells in the hypocotyl under warm conditions: the hypocotyl of the ectopic RBR-GFP expressing line grows significantly longer than that of the WT at warm temperatures. This effect appears to be specific, as epidermal cells in the hypocotyl of the rbr1-2 mutant line were less elongated compared to the WT control. The RBR protein could be detected in epidermal cells of the hypocotyl at both temperatures, regardless of whether they were proliferating or quiescent, supporting its regulatory role in both cell types (Fig. 8). Known thermomorphogenic regulatory genes including the central PIF4 and PIF7 transcription factors together with their target auxin biosynthetic YUCCA genes (YUC1-2 and YUC8-9) were all found to be positively regulated by RBR at the seedling level (Fig. 8). This suggests that RBR mediates the effect of warm temperatures on hypocotyl elongation as a direct or indirect activator of gene expression.

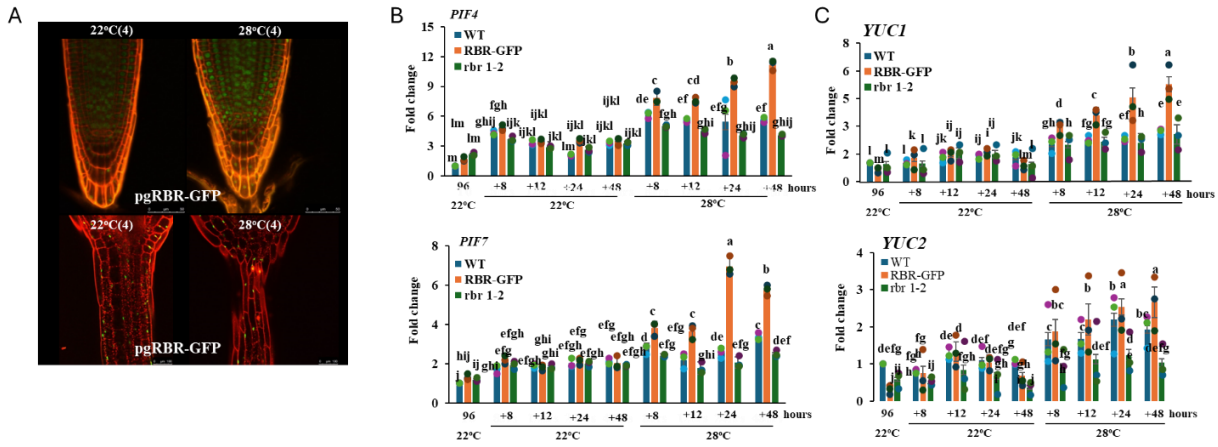

Fig. 8. A) The RBR gene is expressed in both mitotic and post-mitotic cells, and the expression is increased by warm temperatures. Seedlings were grown continuously at 22ºC (control) or transferred from 22ºC to 28ºC (22+28ºC) for the number of days indicated in the parentheses. B-C) Expression of PIF4 and PIF7 (B) and YUC and YUC2 (C) in WT, RBR-GFP and rbr1-2 seedlings at the indicated temperatures and times. T0 represents 96-hour-old seedlings grown at 22oC. These were either kept growing at 22oC or after transferred to 28oC, and samples were taken at 8, 12, 24 and 48 hours afterwards. Values represent fold change normalized to the value of the relevant transcript of the seedlings at T0 (96 hours), which was set arbitrarily at 1. Data are means +/- sd. N=3 biological replicates. Different letters mean statistically significant differences ($p < 0.05$) based on one-way ANOVA analyses with Tukey's HSD post-hoc test.

In summary, analysing the potential role of RBR in the thermoresponse of Arabidopsis seedlings, we made two important discoveries. 1, We demonstrate that an increase in temperature activates cell proliferation in the meristems of both the shoot and root apices soon after the temperature was raised. We suggest that warm temperatures accelerate meristem functions through suppressing the cell cycle inhibitor RBR. 2, We also discovered that RBR not only functions as a cell cycle inhibitor, but also exerts a positive influence on the elongation growth of post-mitotic, quiescent cells under warm conditions. We suggest that RBR mediates the effect of warm temperatures on hypocotyl elongation as a direct or indirect activator of thermomorphogenesis-related gene expression.

Altogether, our data indicate that warm temperatures have a dual effect on RBR activity. It suppresses its canonical cell cycle inhibitor function in both the shoot and root meristems, driving faster growth and development. Additionally, it activates its non-canonical cell elongation regulatory function in the hypocotyl epidermis by increasing, among others, the expression of auxin biosynthetic YUCCA genes. We believe that our results bring thermomorphogenesis research to a new level by connecting its regulation with the cell cycle regulatory CYCD-RBR-E2F pathway. Simultaneously, we have also found new regulatory roles for RBR in the control of auxin-mediated cell elongation and the coordination of plant development with the environment. Based on the novelty of our discoveries, we submitted a manuscript for the Breakthrough section of the journal The Plant Cell (D1 in "Plant science") where it is now under review. The letter of the editor validating the submission is attached. The same manuscript is available for review as a preprint at bioRxiv:
https://www.biorxiv.org/content/10.1101/2024.05.28.596227v1.full.pdf.

To further understand the effect of RBR on temperature enhanced plant growth and gene expression regulation, a genome-wide screening for temperature and RBR-dependent chromatin accessibility changes at gene loci have been attempted using the ATAC seq approach. This was reasoned based on the fact that RBR has functions associated with chromatin organisation (Desvoyes and Gutierrez, 2020). Furthermore, no one has carried out similar analysis to investigate the effect of temperature on genome accessibility. The ATAC seq libraries were made (ATAC-Seg Assay Kit, ActiveMotif, https://www.activemotif.com) from seedlings of four plant lines with two biological replications: Col-0 (control); rbr1-2; RBR::GFP; cycD3 (mutant in the regulatory subunit of the RBR-phosphorylating CDK kinase) grown at two conditions: 22^{o}C (control); 28^{o}C (treatment). The libraries were quality checked and sequenced by Novogen (Cambridge, UK). The same company carried out the bioinformatic analysis to get information about i) the effect of mutations at 22 ^{o}C, ii) the effect of treatment (comparison of 28 ^{o}C treatment to 22 ^{o}C control for each line), iii) to highlight the difference between the responses of the mutant lines to the higher temperature. The libraries passed the quality check. The company carried out a small-scale sequencing first. Based on this, the number and quality of reads was around or over the threshold required for a genome-wide bioinformatic analysis, except for one of the controls (Col-0 at 22 ^{o}C), where it was below. Following discussion with experts, the sequencing was accomplished at the large scale, and enough reads could be achieved to accomplish the bioinformatic analysis. However, it turned out that the two biological replications resulted in rather different gene sets. This resulted in very few statistically significant gene accessibility differences between the samples and those had provided valuable information neither about the effect of the temperature nor the mutations (see Fig. 9. for examples). Even the known thermomorphogenic regulators were not highlighted by the analysis. In summary, the experiment was inconclusive.

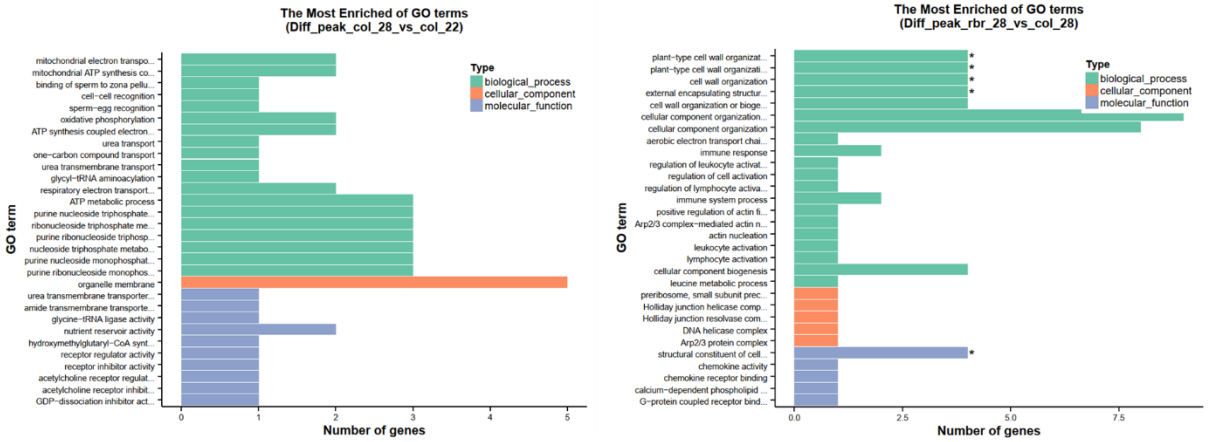

Fig. 9. Gene ontology (GO) term analysis of differentially accessible genes between Col-0 (left) and rbr2-1 (right) plants, respectively, grown at 28^{o}C versus 22^{o}C. Note the low number of genes (x axis) falling into the categories.

3. Thermomorphogenesis in Brachypodium

To access temperature-dependent root growth in Brachypodium, a new culture system was developed allowing the monitoring of root growth (Brachypodium roots did not tolerate the agar solidified medium, unlike Arabidopsis). The system was published in a poster at the XIIIth Congress of Hungarian Society for Plant Biology (24-27.08.2021, Szeged, Hungary).

Moderate increase in shoot length (14 mm vs. 12 mm; 17%) as well as root number (1,7 vs. 1,5 roots/plant; 16%) was observed at 28°C. The most remarkable difference was the increase in the length of the 2nd and 3rd root (37% and 118% respectively) at 28°C summing up 29% increase in total root length of the seedlings at 28°C compared to the control 22°C. Seven Brachypodium distachyon genotypes selected based on their root architecture and drought adaptation response were tested for their thermomorphogenic response in the seedling stage. A high variability was revealed in shoot as well as root responses (Table 1.).

Table 8 Changes in values of root parameters of seedlings of the six representative accessions grown at 22 °C and 28°C

	Bd21 22 C shoot (mm)	total root	root1	root2	root3		Bd21 28 C shoot (mm)	total root	root1	root2	root3	shoot	root
Average:	15,83	30,56	25,44	9,33	9,00	Average:	16,61	30,22	23,48	15,71	15,00	111,59%	94,58%
StDev:	11,03	17,15	18,16	2,21	1,00	StDev:	7,89	16,78	12,71	4,37	5,72		
Average:	24,96	44,46	30,75	19,36	17,00	Average:	28,91	40,73	31,32	18,13	15,50		
StDev:	9,13	14,54	12,57	4,42	1,87	StDev:	7,95	13,83	12,68	2,26	4,72		
	Bd21-3 22 C shoot (mm)	total root	root1	root2	root3		Bd21-3 28 C shoot (mm)	total root	root1	root2	root3		
Average:	18,52	25,04	18,40	9,58	8,50	Average:	23,52	32,05	28,33	15,60			
StDev:	7,80	9,43	11,08	2,84	1,89	StDev:	10,48	11,81	13,86	3,83			
Average:	20,74	27,17	22,74	8,89	9,00	Average:	16,27	21,00	19,65	11,00	7,00		
StDev:	6,37	10,99	13,27	3,03	0,00	StDev:	9,13	9,33	8,20	3,00	0,00		
	Bd3-1 22 C shoot (mm)	total root	root1	root2			Bd3-1 28 C shoot (mm)	total root	root1	root2	root3		
Average:	20,64	22,72	21,92	10,00		Average:	31,70	33,55	33,55	21,50	18,00		
StDev:	9,09	11,98	12,36	1,00		StDev:	11,25	14,98	14,98	6,50	0,00		
Average:	24,62	23,29	22,57	7,50		Average:	25,41	24,00	20,82	20,50	13,00		
StDev:	7,40	14,24	14,55	3,50		StDev:	11,33	12,62	8,93	2,50	0,00		
	Bd18-1 22 C shoot (mm)	total root	root1				Bd18-1 28 C shoot (mm)	total root	root1			shoot	root
Average:	11,12	35,29	35,29			Average:	29,43	44,71	44,71			197,60%	127,01%
StDev:	3,91	8,07	8,07			StDev:	9,51	14,15	14,15				
Average:	16,88	33,41	33,41			Average:	25,90	42,55	42,55				
StDev:	8,48	15,58	15,58			StDev:	11,75	18,94	18,94				
	Bd1-1 22 C shoot (mm)	total root	root1				Bd1-1 28 C shoot (mm)	total root	root1	root2		shoot	root
Average:	10,14	26,73	26,73			Average:	18,96	32,58	32,08	6,00		181,60%	133,38%
StDev:	2,75	4,83	4,83			StDev:	6,17	13,92	14,62	2,00			
Average:	11,36	26,61	26,61			Average:	20,07	38,56	38,56				
StDev:	4,35	8,01	8,01			StDev:	5,70	15,33	15,33				
	GAZ6 22 C shoot (mm)	total root	root1	root2	root3		GAZ6 28 C shoot (mm)	total root	root1	root2	root3	shoot	root
Average:	22,85	42,11	34,93	20,11		Average:	34,50	56,60	50,43	22,17	23,00	131,84%	110,80%
StDev:	6,38	14,38	14,79	4,41		StDev:	8,29	11,84	14,70	11,08	0,00		
Average:	21,87	34,70	34,26	10,00		Average:	24,46	28,50	27,92	15,00			
StDev:	7,18	13,87	14,44	0,00		StDev:	11,31	14,38	14,53	0,00			

For gene expression analysis root segment samples (root tip, elongation zone, differentiated zone etc.) of young Brachypodium seedlings grown at 22°C and 28°C were collected. Total cellular RNA was purified of each of the 12 segments. Based on the regulation of adventitious root formation we investigated the expression of 8 members of the LATERAL ORGAN BOUNDARY (LOB)-domain gene family qPCR experiments revealed that five of them exhibited significant expression differences in roots developed at 22°C or 28°C, respectively, and also with or without the reorientation of the roots by 90^{o}. We also investigated the potential link between the circadian clock and thermomorphogenesis due to the well-documented root growth pattern during the night. Our initial investigation involved examining the expression of core clock genes in the root system of Brachypodium to determine if they followed the expected regulatory pattern. We observed that while most core clock genes (LHY, TOC1, PRR95, and GI) showed reduced amplitude of transcript level changes in roots compared to aerial parts, their circadian cycling of expression remained consistent. In contrast, ELF3 and ELF4-like genes did not display circadian expression in the roots of this monocot model. Furthermore, we found that stress influenced circadian gene expression in the roots. These novel findings have been published as part of a research paper (Gombos *et al.*, 2023).

After that the link between the cell cycle and thermomorphogenesis has been established in Arabidopsis, the expression profile of several known core cell-cycle genes and morphogenesis regulatory genes were followed in Brachypodium. We selected the available Brachypodium orthologues of those genes which exhibited reasonable changes in the dicot model system, in Arabidopsis. CDK-B1.1, CYC-A3.1, CYC-D3.1, MCM3, ORC2, FBL17 and HY5.
Significant changes in the expression profile of six genes (out of seven) were observed, however most of them are below 2-fold transcript level variation.
The most remarkable differences were the followings:
- ORC2: 90° reorientation of the roots resulted in increased expression in the elongation and differentiated zone of the roots at 28°C, whereas no effect was observed at 22°C;
- HY5: the gene is expressed at the highest level always in the elongating zone of the root (2nd sample) at both temperatures (2-3 fold higher than in zone 1 and 3), however the level of mRNA is higher at 28°C than at 22°C in every sample and the difference is the most pronounced when the roots are transferred from 22°C to 28°C.

We are currently writing a manuscript on the observed temperature-dependent gene changes in Brachypodium roots. The manuscript is at the finalization stage at the time of preparing this report.

4. Summary

We faced several problems during the project such as the COVID pandemic, difficulties to set up and use the plant phenotyping system, frequent change in the research personnel, etc. Furthermore, despite of supporting preliminary data, the involvement of the RLCKVI_A2 kinase in thermomorphogenic hypocotyl elongation could not be validated. Nevertheless, refocusing our research efforts on tasks that promised novel findings contributed to or resulted in several publications listed below:

Papers:

Shiekh R, Nagy F, Kaszler N, Domonkos I, Gombos M, Molnár E, Pettkó-Szandtner A, Bögre L, Fehér A, Magyar Z. 2024. Retinoblastoma-related (RBR) has both canonical and non-canonical regulatory functions during thermo-morphogenic responses in Arabidopsis seedlings. bioRxiv. https://www.biorxiv.org/content/10.1101/2024.05.28.596227v1
Preprint. Under review in The Plant Cell (for the letter acknowledging submission see further)

Kenesi E., Beöthy-Fehér O., Szőllősi R, Domonkos I., Valkai I, Fehér A. (2024) The REPLUMLESS transcription factor controls the expression of the RECEPTOR-LIKE CYTOPLASMIC KINASE VI_A2 gene involved in shoot and fruit patterning of Arabidopsis thaliana. Preprint.org. Preprints ID: preprints-111276. Under review in International Journal of Molecular Sciences. (for the letter acknowledging submission see further)

Szécsényi M., Kiss E., Zombori Z., Gombos M., Sándor G., Györgyey J. Natural variation in root architecture of Brachypodium distachyon accessions during moderate water-deficit stress and temperature shift. Manuscript in preparation

Lang L., Pettkó-Szandtner A., Elbasi H.T., Takatsuka H., Nomoto Y., Zaki A., Dorokhov S., De Jaeger G., Eeckhout D., Ito M., Magyar Z., Bögre L., Heese M., Schnittger A. The DREAM complex represses growth in response to DNA damage in Arabidopsis. LIFE SCIENCE ALLIANCE (2575-1077): 4 12 Paper 202101141. (2021) https://www.life-science-alliance.org/content/4/12/e202101141

Gombos M, Hapek N, Kozma-Bognár L, Grezal G, Zombori Z, Kiss E, Györgyey J. 2023. Limited water stress modulates expression of circadian clock genes in Brachypodium distachyon roots. Scientific Reports 13, 1241. https://www.nature.com/articles/s41598-022-27287-4

Lectures/Posters

Sheikh, R., Nagy, F., Györgyey, J., Fehér, A. and Magyar Z. The RETINOBLASTOMA-RELATED (RBR) in complex with the E2FB transcription factor could regulate thermomorphogenesis in Arabidopsis. Poster abstract, ÖSSZEFOGLALÓ KÖTET, XIIIth Congress of Hungarian Society for Plant Biology, 24-27.08.2021, Szeged, Hungary, ISBN 978-615-01-2350-9, p.88
https://drive.google.com/file/d/16EFwR394IiUOMzFqc1IJGGNwKcSF0-T0/view?usp=sharing

Kiss E., Gombos M., Fehér A., Györgyey J. Developing a modell system for monitoring thermomorphogenetic changes of the root architecture in the model monocot Brachypodium distachyon. Poster abstract, ÖSSZEFOGLALÓ KÖTET, XIIIth Congress of Hungarian Society for Plant Biology, 24-27.08.2021, Szeged, Hungary, ISBN 978-615-01-2350-9, p.73
https://drive.google.com/file/d/16EFwR394IiUOMzFqc1IJGGNwKcSF0-T0/view?usp=sharing

Fehér A., Valkai I., Lajkó DB., Kenesi B., Ménesi D., Borbély P., Bodai L., Durr J. ROP GTPáz aktivált kináz jelátvitel növényekben. Lecture abstract, ÖSSZEFOGLALÓ KÖTET, XIIIth Congress of Hungarian Society for Plant Biology, 24-27.08.2021, Szeged, Hungary, ISBN 978-615-01-2350-9, p. 44https://drive.google.com/file/d/16EFwR394IiUOMzFqc1IJGGNwKcSF0-T0/view?usp=sharing

Hlacs A, Gombos M, Borics A, Györgyey J (2022): Predicted phosphorilation and dimerization of certain LBD transcription factors expressed in Brachypodium distachyon based on molecular dynamics; Straub Days, ELKH Szegedi Biológiai Kutatóközpont, 2022. 05. 25-27, Szeged

Rasik Shiekh Bin Hamid, Fruzsina Nagy, Magdolna Gombos, Aladár Pettkó-Szandtner, Attila Fehér, Zoltán Magyar (2022) The RETINOBLASTOMA-RELATED (RBR) controls thermomorphogenesis by regulating cell proliferation and cell enlargement in a temperature dependent manner. Straub Days, ELKH Szegedi Biológiai Kutatóközpont, 2022. 05. 25-27, Szeged

Shiekh R, Nagy F, Gombos M, Pettkó-Szandtner A, Fehér A, Magyar Z.: The RETINOBLASTOMA-RELATED (RBR) controls thermomorphogenesis by regulating cell proliferation and cell enlargement in a temperature dependent manner. Plant Biology Europe 2023, Marseille, France, https://europlantbiology2023.org/wp-content/uploads/2023/07/PBE2023-Abstract-Book.pdf, 2023

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Lang L, Pettkó-Szandtner A, Tunçay Elbaşı H, *et al.* 2021. The DREAM complex represses growth in response to DNA damage in Arabidopsis. Life Science Alliance **4**, e202101141.

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Yang X, Dong G, Palaniappan K, Mi G, Baskin TI. 2017. Temperature-compensated cell production rate and elongation zone length in the root of Arabidopsis thaliana. Plant, Cell & Environment **40**, 264–276.

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On 20-Jun-2024, the manuscript entitled "Retinoblastoma-related (RBR) has both canonical and non-canonical regulatory functions during thermo-morphogenic responses in Arabidopsis seedlings" by Zoltan Magyar, Rasik Shiek, Fruzsina Nagy, Nikolett Kaszler, Ildiko Domonkos, Magdolna Gombos, Eszter Molnár, Aladár Pettko-Szandtner, László Bögre, and Attila Fehér was submitted to The Plant Cell by the corresponding author.

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Authors: Erzsébet Kenesi, Orsolya Beöthy-Fehér, Réka Szőllősi, Ildikó Domonkos, Ildikó Valkai, Attila Fehér *
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