RETINOBLASTOMA-RELATED regulates cell size of mitotic and post-mitotic cells under standard and elevated temperatures in *Arabidopsis thaliana*

Ph.D. Dissertation

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INTRODUCTION

Climate change presents a significant challenge to current and future crop production efficiency and food security (Ray *et al.* 2019; Ortiz-Bobea *et al.* 2021; Mirón *et al.* 2023). Temperature exerts a considerable influence on plant development, growth, metabolism, and defence mechanisms (Lippmann *et al.*, 2019), thereby significantly affecting the distribution of optimal crop production regions (Anderson *et al.*, 2020). As sessile organisms, plants lack homeostatic mechanisms to regulate body temperature. They have evolved sophisticated regulatory mechanisms to adapt to varying temperatures, ensuring enhanced survival and reproduction, as well as maintaining growth and development (McClung et al., 2016). Thermomorphogenesis, the response of plants to warm ambient temperature is characterized by elongation of the hypocotyl, stem, petiole, and primary root, along with leaf hyponasty and reduce surface area (Quint *et al.*, 2016; Casal and Balasubramanian, 2019; Lippmann *et al.*, 2019; Wang and Zhu, 2022), which collectively contribute to enhanced evaporative leaf cooling (Crawford *et al.*, 2012; Bridge *et al.*, 2013; Park *et al.*, 2019). Our understanding of the molecular players involved in this process primarily stems from studies on hypocotyl elongation in Arabidopsis seedlings grown at 27-29°C (thermomorphogenesis) compared to 22-24°C (typical morphogenesis). To initiate an appropriate response to

fluctuating temperatures, plants require precise mechanisms for detecting temperature changes. Recent studies have identified various ambient temperature sensors and elucidated their mechanisms for temperature detection in plants. Phytochrome B (PhyB), a photoreceptor comprising an open-chain tetrapyrrole chromophore, has been identified as a temperature sensor that regulates various aspects of thermomorphogenesis acceleration during flowering, senescence, and architectural changes (Jung et al., 2016; Quint et al., 2016). Recent research has identified a discrete RNA-based thermo "switch" in plants. Ribosome profiling was employed to identify genes that showed elevated translational efficiency upon exposure to warm ambient temperatures (27°C; Chung et al. 2020). Among thesegenes, *PHYTOCHROME INTERACTING FACTOR 7* (*PIF7*) was notably observed to have enhanced translation. PIF7 is a pivotal transcription factor involved in regulating morphological changes at high temperatures (Chung et al. 2020, Fiorucci et al., 2020). A significant advancement in understanding the mechanism of thermo-morphogenesis in response to moderately increased temperatures was the identification of the basic helix-loop-helix (bHLH) transcription factor *PHYTOCHROME INTERACTING FACTOR 4* (*PIF4*), a crucial transcription factor that facilitates thermomorphogenesis (Koini et al., 2009; Sun et al., 2012). During ambient temperature-dependent hypocotyl elongation, auxin synthesis and

signalling are among the primary targets of PIF4 (Koini *et al.*, 2009; Franklin *et al.*, 2011; Sun *et al.*, 2012; Bianchimano *et al.*, 2023). PIF4 directly upregulates genes encoding *YUCCA8* (*YUC8*), *INDOLE-3-ACETIC ACID INDUCIBLE 19* (*IAA19*), and *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*).

In comparison to the hypocotyl and other aboveground plant organs, the thermomorphogenic response of the root has been less investigated (Fonseca de Lima *et al.*, 2021). Under laboratory conditions, a moderate temperature of 26-29°C is sufficient to stimulate the growth of the primary root of Arabidopsis, a phenomenon that is mainly controlled by auxin (Fonseca de Lima *et al.*, 2021; Ai *et al.*, 2023; Bianchimano *et al.*, 2023); however, in long-term responses, the primary role of brassinosteroids has been implicated (Martins *et al.*, 2017). While the thermo-regulated elongation of the Arabidopsis hypocotyl primarily depends on auxin- and brassinosteroid-dependent increases in cell size and less on cell division (Bellstaedt *et al.*, 2020), a recent study has reported that an auxin-mediated increase in meristem cell division activity is primary and predominant in the thermosensory response of the roots (Ai *et al.*, 2023). However, the contribution of auxin and/or brassinosteroid-dependent cell elongation, particularly under prolonged high-temperature conditions, cannot be excluded from the root (Martins *et al.*, 2017; Yang *et al.*, 2017).

Proliferation in plants occurs in meristems, which are essential for plant growth and development. The shoot apical meristem (SAM) contains stem cells at the tip within the central zone (CZ), surrounded by rapidly dividing transit amplifying (TA) cells that can differentiate. Stem cells are maintained in limited numbers, with progeny moving toward the periphery during development. The root grows continuously due to stem cells in the root apical meristem (RAM). In Arabidopsis, all cell layers originate from specific initials in a radial pattern of clonal cell files. The mechanism linking thermal regulation of auxin signalling to cell division in roots remains unclear, while the shoot's thermomorphogenic response rarely considers accelerated cell divisions. If elevated temperatures affect meristem activity, it likely increases cells entering the cell cycle. The plant cell cycle is controlled by conserved mechanisms, particularly the RETINOBLASTOMA-E2F pathway (Magyar Z., 2008; Berckmans, B., & De Veylder, L. 2009).

The entry into the cell cycle is governed by conserved molecular mechanisms, including RETINOBLASTOMA (RB) protein, which regulates cell cycle progression through interaction with E2F/DP transcription factors (Henley and Dick, 2012). Arabidopsis contains a single *RETINOBLASTOMA-RELATED* (*RBR*) gene, linking environmental signals to cell proliferation and

differentiation (Harashima and Sugimoto, 2016). RBR interacts with three E2F transcription factors: E2FA, E2FB, and E2FC. E2Fs must form heterodimers with DIMERIZATION PARTNER proteins (DPA and DPB – Magyar et al., 2000). Studies categorized these E2Fs as activators (E2FA and E2FB) or repressor (E2FC). E2FA promotes cell proliferation in S-phase cells (De Veylder et al., 2002), while E2FB facilitates G1/S and G2/M transitions throughout the cell cycle. Like animal activators E2F1-3, the ectopic expression of E2FB induces cell proliferation without the growth promoting hormone auxin, supporting its role as a cell cycle activator (Magyar et al., 2005).

OBJECTIVES

During my thesis work I focused on two main topics:

1, Previous research on Arabidopsis thaliana has underscored the influence of warm ambient temperatures on overall growth and accelerated development, primarily attributing these effects to cell elongation. This study investigates how warm temperatures affect meristematic activity in *Arabidopsis thaliana* during thermo-morphogenesis, focusing on the role of RBR, a cell cycle inhibitor. By modulating RBR levels, we aim to assess any morphological, cellular and molecular changes its impact on phosphorylation, cell cycle dynamics, and key thermo-morphogenic genes in shoot and root meristems.

2, In our study of thermomorphogenesis, the Retinoblastoma-Related (RBR) protein regulates post-mitotic cell size in a temperature-dependent manner. We hypothesized that RBR dosage in Arabidopsis affects cell size in vivo. Using transgenic plants with modified RBR levels, we observed cell size changes from embryogenesis through organ development. Reduced RBR levels formed clusters of small stomatal meristemoids, confirming its influence on cell size. We aimed to understand this mechanism by analysing regulatory genes in stomatal development's proliferation-differentiation balance.

Materials and Methods:

Plant material and growth conditions:

WT Col-0, RBR-GFP (Magyar et al., 2012), CYCB1;2-YFP (Iwata et al., 2011), *rbr1-2* mutant (Nowack et al., 2006), *e2fa-1* and *e2fb-2* mutant (Leviczky et al., 2019), triple *cycd3;1-3* mutant (Dewitte et al., 2007). At constant temperatures of 22ºC or 28ºC under long day conditions (16 h light/8 h dark) and with 100 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) from white fluorescent lamps.

Microscopy

Confocal laser microscopy (SP5, Leica). Seedlings were grown on vertically oriented plates, and roots were stained with propidium iodide (PI – 20 µg/mL) and photographed

afterwards. Cell length was measured by using Image J software.

JEOL JSM-7100F/LV scanning electron microscope Young seedlings were vacuum infiltrated and fixed with 100% methanol for 20 min, dehydrated in 100% ethanol for 30 min and then in fresh 100% ethanol overnight, dried and mounted on SEM stubs and observed in a JEOL JSM-7100F/LV scanning electron microscope in low-vacuum mode.

Dissecting the mature embryos

Mature dried seeds of WT and transgenic lines (RBR-GFP and *rbr1-2*) were imbibed for 1 h and dissected under the stereo-microscope and analysed under confocal laser microscope using PI.

RT-qPCR

RNA was extracted from young seedlings in the thermomorphogenesis experiment while young cotyledons and Ist leaf pair was used to extract RNA for the cell size experiment. This was done by using a CTAB-LiCl method described as Jaakola et al., 2001. RNA samples were treated with DNase1 (ThermoScientific #EN0521) according to the manufacturer's protocol.

Summary of the results:

In light of the effects of climate change on plant diversity and agriculture, it is imperative to comprehend the mechanisms by which plants adapt to increased temperatures, as this understanding is vital for ensuring food security. As sessile organisms, plants have developed strategies such as thermomorphogenesis, which involves altering growth and morphology in response to temperature fluctuations.

In plants, specific regions known as meristems are responsible for regulating growth and development. These regions are the primary sites of cell proliferation, which serves as the major driving force behind plant growth. While elevated temperatures accelerate growth and induce morphological changes like elongation of hypocotyl, petiole, and root, the role of meristems in these alterations remains less understood.

Through the analysis of meristematic function in young Arabidopsis seedlings shortly after an increase in temperature, we identified an activation of cell proliferation in both the shoot and root apices. This activation led to accelerated leaf development and enhanced root growth. The cell cycle regulatory genes, including G1-specific *CYCLIN D3;1* (*CYCD3;1*) and *CYCLIN A3;1* (*CYCA3;1*), were rapidly activated. These

cyclins serve as the regulatory subunits of the RETINOBLASTOMA-RELATED (RBR)-kinase, CYCLIN-DEPENDENT-KINASE A;1 (CDKA;1). Notably, RBR was observed to undergo prompt phosphorylation following temperature shifts. RBR functions as a cell cycle inhibitor through E2F transcription factors. RBR phosphorylation activates E2Fs, which trigger S and G2 phase cell cycle genes under elevated temperatures. By modulating the RBR level in plants through the use of two transgenic lines that express either ectopic RBR (RBR-GFP) or reduced *rbr1-2* lines, respectively, we can either enhance or alleviate the impact of elevated temperatures on meristematic function in a concentration dependent manner. In contrast to meristems, the hypocotyl predominantly consists of post-mitotic cells, and elevated temperatures promote their elongation growth. Notably, an increased level of ectopic RBR further enhances the elongation growth of these cells, whereas a reduced RBR level exerts the opposite effect, resulting in smaller epidermal cells compared to the WT control. Surprisingly, key thermomorphogenic regulatory genes, such as *PHYTOCHROME INTERACTING FACTOR 4* and *7* (*PIF4*, and *PIF7*), along with downstream targets, auxin biosynthetic *YUCCA* (such as *YUC8*) are induced by RBR. This indicates that RBR positively regulates their expression in a concentration-dependent manner under elevated temperature conditions. The ectopic RBR-mediated effect

on hypocotyl elongation was significantly influenced by the E2FB mutation, while E2FA loss showed no effect under elevated temperature conditions. Therefore, we suggest that RBR controls hypocotyl elongation in conjunction with E2FB under conditions of elevated temperature. The mutation of the entire CYCD3 subclass demonstrates that the meristematic inhibitory and elongation-promoting functions of RBR are negatively regulated by CYCD3. Consequently, the *cycd3;1-3* triple mutant exhibits phenotypic similarities to the ectopic RBR-GFP expressing line, as evidenced by reduced root lengths and elongated hypocotyls at elevated temperatures compared to the wild-type (WT) control. Furthermore, the *cycd3;1-3* mutant exhibits diminished expression of cell cycle genes, such as *ORC2* and *CDKB1;1*, while demonstrating increased induction of thermomorphogenic genes, including *PIF4*, *PIF7*, *YUC2*, and *YUC8*, at elevated temperatures. This observation substantiates that CYCD3;1 and its closely related proteins promotes cell proliferation through RBR inactivation, whereas its absence redirects growth towards cell elongation, akin to the effects observed with RBR overexpression.

We also demonstrated that RBR serves as a regulator of cell size in developing Arabidopsis seedlings and embryos, operating in a concentration-dependent manner. Mitotic cells in plant tissues undergo proliferation either symmetrically or asymmetrically,

contingent upon their positional context and developmental fate. In the root meristem, progenitor cells that divide symmetrically exhibit significant size variation influenced by the level of RBR. Specifically, an elevated RBR level in the ectopic RBR-GFP expressing line results in enlarged cells, whereas a reduced RBR level in the *rbr1-2* mutant leads to smaller cells compared to the WT control. We propose that RBR regulates the expression of cell cycle genes involved in the transitions from the G1 to S phase and from the G2 to M phase, thereby influencing the duration of the G1 and G2 phases, which have been identified as crucial for cell size control in plants (D'Ario et al., 2021; Nomoto et al., 2022).

In cotyledon and leaf epidermis, stomatal meristemoid cells undergo several rounds of asymmetric division before transitioning to symmetric division, ultimately resulting in the formation of guard cells. The reduction of RBR levels in the *rbr1-2* mutant led to an overproliferation of meristemoid cells, which notably became extremely small in size. Conversely, an increase in RBR levels resulted in decreased proliferation activity, accompanied by an enlargement in cell size. Our data indicate that RBR functions as the nuclear factor responsible for determining the cell size threshold in meristemoid cells. In support of this hypothesis, the RBR dosage modulates stomatal lineage progression by differentially regulating cell-cycle genes, such as *ORC2* and *CDKB1;1*, as well as bHLH transcription factors,

primarily *SPCH* and *MUTE*. In *rbr1-2* mutants, elevated levels of SPCH and its downstream target CYCD3;1 led to excessive asymmetric divisions. Conversely, overexpression of RBR-GFP enhances the induction of MUTE and its cyclin-D targets, CYCD5;1 and CYCD7;1, thereby accelerating the formation of symmetric guard cells.

In summary, the cell cycle inhibitor RBR regulates cell size in both mitotically active cells within meristems and young developing organs, as well as in post-mitotic cells under conditions of elevated temperature. Our data suggest that RBR regulates both cell number and size through distinct mechanisms. A deeper understanding of these RBR-centred molecular pathways offers the potential to influence plant growth, which is beneficial for plant breeding programs. Further investigation is necessary to elucidate the precise molecular mechanisms underlying these RBR-mediated processes.

List of Publications:

1, Hamid RSB, Nagy F, Kaszler N, Domonkos I, Gombos M, Marton A, Vizler Cs, Molnár E, Pettkó-Szandtner A, Bögre L, Fehér A., Magyar Z. (2025) RETINOBLASTOMA-RELATED Has Both Canonical and Noncanonical Regulatory Functions During Thermo-Morphogenesis Responses in Arabidopsis Seedlings. **PLANT CELL AND ENVIRONMENT** 48: (2) 1217-1231. doi: 10.1111/pce.15202. Epub 2024 Oct 17 MTMT: 35472739

2, Fehér A, Hamid RSB, Magyar Z. (2025) How Do Arabidopsis Seedlings Sense and React to Increasing Ambient Temperatures? **Plants – Basel,** 14(2):248. https://doi.org/10.3390/plants14020248
MTMT: 35766994

Co-Author wavier form

As the corresponding and/or contributing author of the mentioned publications, I declare that the authors have no conflict of interest related to this study. l also declare that the Ph.D. candidate Shiekh Rasik Bin Hamid worked under my supervision and his contribution was prominent in obtaining the results, and his first author publication was not used for Ph.D. defense by any of the co-authors.

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