Identification of novel and ABA-regulated miRNA and characterization of its target gene-AtBro1 upon growth and abiotic stress response in *Arabidopsis thaliana*

Identyfikacja nowego i regulowanego przez ABA miRNA oraz charakterystyka jego genu docelowego - AtBro1 w procesach wzrostu i w odpowiedzi na stres abiotyczny u *Arabidopsis thaliana*

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STRESZCZENIE

Wiele czynników środowiskowych, takich jak podwyższona temperatura, zasolenie i susza, mają wpływ na wzrost i produktywność upraw. Kluczowym szlakiem sygnałowym uczestniczącym w regulacji odpowiedzi roślin na stres jest rdzeniowa sygnalizacja kwasu absysynowego (ABA). ABA kontroluje reakcje roślin poprzez regulację ekspresji genów, także z udziałem ścieżek zależnych od miRNA. miRNA biorą udział w różnych procesach biologicznych, w tym w reakcjach adaptacyjnych na stres abiotyczny.

Celem mojej pracy doktorskiej jest identyfikacja nowych miRNA zaangażowanych w regulację ekspresji genów w odpowiedzi na ABA. Aby zrealizować postawiony cel badania prowadzono z zastosowaniem rośliny modelowej *Arabidopsis thalina* formy dzikiej (WT) oraz mutantów szlaku sygnałowego ABA: *abi1td*, *mkkk17* i *mkkk18*. Analizy z wykorzystaniem RNAseq umożliwiły identyfikację 10 nowych miRNA w odpowiedzi na ABA (ath-miRn-1, ath-miRn-2, ath-miRn-3, ath-miRn-4, ath-miRn-5, ath-miRn-6, ath-miRn-7, ath-miRn-8, ath-miRn-9 and ath-miRn-10) u formy dzikiej *A.thaliana* oraz 1 nowy miRNA u mutanta *mkkk17*. Ponadto, analiza wyników ujawniła, że trzy, siedem i dziewięć znanych miRNA ulegało różnej ekspresji w odpowiedzi na ABA u mutantów *abi1td*, *mkkk17* i *mkkk18*. Wyniki sekwencjonowania zostały potwierdzone za pomocą ilościowego RT-PCR we wszystkich badanych genotypach. By zidentyfikować geny docelowe i potencjalne miejsca cięcia dla nowozidentyfikowanych miRNA wykorzystano psRNA-Target oraz 5′ RLM-RACE. Analiza ontologii wykazała, że potencjalne geny docelowe znanych i nowych miRNA reagujących na ABA są zaangażowane w różne procesy komórkowe w roślinach, w tym rozwój i ruchy aparatów szparkowych. Wyniki te sugerują, że wiele zidentyfikowanych miRNA odgrywa ważną rolę w odpowiedziach roślin na stres środowiskowy i może pełnić wspólne funkcje regulacyjne w szlaku sygnałowym ABA.

W kolejnym etapie badania koncentrowały się na charakterystyce nieznanego białka BRO1, zawierającego domenę podobną do BRO (AT1G73390), które jest celem jednego z nowo zidentyfikowanych miRNA, ath-miRn-1. W toku analiz wykazano, że w odpowiedzi na zasolenie, ABA i mannitol transkrypt *BRO1* ulegał indukcji. Transgeniczne linie *A.thaliana* z nadekspresją BRO1 wykazywały silną tolerancję na suszę i stres solny wskazując, że BRO1 reguluje reakcje odpornościowe u roślin. Promotor *AtBRO1* w fuzji z GUS wykazywał ekspresję głównie w liściach rozety i kwiatach, zwłaszcza w pylnikach. Analiza lokalizacji subkomórkowej BRO1::GFP wykazała, że białko to lokalizuje się przy błonie cytoplazmatycznej protoplastu. Wyniki analizy transkyptomicznej mutanta typu nokaut w genie *BRO1, bro1-1,* w odniesieniu do linii dzikiej Arabidopsis, wykazały zmiany w ekspresji wielu genów, o których wiadomo, że są zaangażowane w odpowiedź roślin na stres. Stwierdzono, że BRO1 może odgrywać ważną rolę w regulacji odpowiedzi transkrypcyjnej na ABA i indukcji odpowiedzi roślin na stres abiotyczny.

Podsumowując, wyniki badań przedstawione w prezentowanej rozprawie, umożliwiły poznanie nowych mechanizmów regulujących odpowiedź roślin na stres abiotyczny i powiązanych z sygnalizacją ABA. Najważniejszym wynikiem pracy jest identyfikacja aż 10 nieznanych miRNA i głębsza charakterystyka funkcjonowania jednego ze z nich - ath-miRn-1 – w powiązaniu z identyfikacją nowego efektora w reakcji roślin na stres i nieznanego dotąd *BRO1*. Wyniki uzyskane w ramach pracy rzucają światło na mechanizmy leżące u podstaw tolerancji na stres.

ABSTRACT

Crop growth and productivity can be perturbed by multiple environmental factors, such as climate change, which are associated with higher ambient temperatures, increased salinity and harsh drought. The core abscisic acid (ABA) signaling pathway regulates plant growth and development by controlling gene expression. ABA also affects the abundance of several microRNAs (miRNAs), which control downstream genes in plants. miRNAs are involved in various biological processes, including adaptive responses to abiotic stress.

The first aim of my doctoral study was to identify novel miRNAs involved in the response to ABA; such ABA-responsive miRNAs were identified by small-RNA sequencing in wild-type (WT) *Arabidopsis thaliana*, as well as in *abi1td*, *mkkk17* and *mkkk18* mutants. I identified 10 novel miRNAs in WT after ABA treatment, while in the *abi1td*, *mkkk17* and *mkkk18* mutants, three, seven and nine known miRNAs, respectively, were differentially expressed after ABA treatment. One novel miRNA (*ath-miRn-8*) was differentially expressed in the *mkkk17* mutant. I validated the sequencing results by quantitative RT-PCR of several known and novel miRNAs in all genotypes and predicted potential target genes of the miRNA panel using psRNATarget. Of the predicted targets of novel miRNAs, I verified cleavage sites in target genes *AT1G73390* (encoding a Bro1-like domain-containing protein) for *ath-miRn-1*, *AT5G40550* (*SGF29b*) for *ath-miRn-2*, *AT3G14070* (*CAX9*) for *ath-miRn-4*, *AT1G56650* (*MYB75*), *AT5G58490* for *ath-miRn-6*, *AT3G15570* (encoding a phototropic-responsive *NPH3* family protein) for *ath-miRn-8*, and *AT2G29140* (*PUM3*) for *ath-miRn-9* using 5′ RLM-RACE.

Gene Ontology analyses showed the potential target genes of ABA-responsive known and novel miRNAs to be involved in diverse cellular processes in plants, including development and stomatal movement. These results suggest that a number of the identified miRNAs have

important roles in plant responses to environmental stress and might have common regulatory functions in the core ABA signaling pathway.

The next part of the study focused on characterization of the novel miRNA target gene, *AtBro1* (initially known as *AT1G73390*; see above), and its role in the response to abiotic stress in Arabidopsis. *AtBro1* was upregulated in plants treated with salt, ABA and mannitol. *AtBro1*-overexpression lines demonstrated robust tolerance to drought and salt stress. Furthermore, ABA stimulated resistance responses in a loss-of-function *bro1* mutant and *AtBro1* positively regulated drought resistance in Arabidopsis. When the *AtBro1* promoter was used to drive the β-glucuronidase (GUS) gene in transgenic plants, GUS was expressed mainly in rosette leaves and floral clusters, especially in anthers. Introduction of an AtBro1-GFP fusion protein construct into Arabidopsis protoplasts showed that AtBro1 protein is localized in the plasma membrane. Global RNA-sequencing analysis showed that early transcriptional responses prompted by ABA treatment exhibit specific quantitative differences at different time points, suggesting that ABA stimulates resistance responses in *bro1* mutant plants. Additionally, transcript levels of *MOP9*.5, *MRD1*, *HEI10* and *MIOX4* were altered in loss-of-function mutant plants in response to different stress conditions. Collectively, it can be concluded that AtBro1 likely plays a significant role in the regulation of the plant transcriptional response to ABA and the induction of resistance responses to abiotic stress.

In conclusion, the results presented have allowed the identification of new mechanisms regulating the response of plants to abiotic stress and related to ABA signaling. The most important result is the identification of 10 unknown miRNAs and a deeper characterization of the function of one of them - ath-miRn-1 - in connection with the identification of a new effector in the plant response to stress - the previously unknown BRO1. The results of this work will shed light on the mechanisms underlying stress tolerance in plants.

AIM OF THE STUDY

The main goal of my doctoral dissertation was to verify the hypothesis that abscisic acid (ABA) affects the abundance of microRNAs (miRNAs) that control downstream genes in the core ABA signaling pathway and ABA-regulated MAPK cascade, and to characterize a target gene of one of the novel miRNAs identified in Arabidopsis. Accordingly, the experimental work plan of my PhD thesis was divided into two parts:

1. Identification of novel miRNAs in the ABA signaling pathway (**Publication # 1**).
    - Using small-RNA next-generation sequencing, identify novel ABA-responsive miRNAs involved in the ABA signaling pathway in Arabidopsis WT Col-0, *abi1td*, *mkkk17* and *mkkk18* mutant's lines.
    - Confirm novel miRNA transcripts in all genotypes by qRT-PCR using TaqMan miRNA assays.
    - Predict novel ABA-responsive miRNA target genes using the psRNATarget database.
    - Identify cleavage sites of novel miRNAs in target genes using 5′ RLM-RACE.
    - Check expression levels of novel miRNA target genes by qRT-PCR.
2. Characterization of the target gene, *AtBro1*, of a novel miRNA (*ath-miRn-1*; identified in part 1), which is suppressed by ABA treatment in WT and other genotypes. Investigation of the involvement of *AtBro1* in the response to various abiotic stresses in WT and other Arabidopsis genotypes. AtBro1 protein function was completely unknown and uncharacterized prior to this work (**Publication # 2**):
    - Identify paralogs and orthologs of *AtBro1* by bioinformatics.
    - Determine the *AtBro1* expression pattern in response to multiple stress factors.
    - Analyze the spatial and temporal expression patterns of *AtBro1* during seedling development in WT Arabidopsis plants by qRT-PCR.

- Analyze the core and *cis*-regulatory elements in the upstream region of the *AtBro1* gene and carry out GUS expression analysis in plants.
- Investigate the subcellular localization of an AtBro1-GFP C-terminal fusion protein in Arabidopsis protoplasts by confocal microscopy.
- Analyze the *AtBro1* T-DNA insertion line, as well as *AtBro1*-overexpression and complementary lines.
- Investigate the physiological and biological function of AtBro1 in plants subjected to ABA, mannitol, sodium chloride and drought stress.
- Examine the effects of AtBro1 on the expression of signaling pathway and biosynthesis-related genes.
- Determine the effect of AtBro1 on global gene regulation by RNA-seq analyses.
- Investigate, and validate by qRT-PCR, selected genes shown to be differentially expressed by RNA-seq in WT Arabidopsis and in mutant and overexpression lines.

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LIST OF SCIENTIFIC WORKS INCLUDED IN THE DISSERTATION

The results of the experimental work in this thesis are described in the following research articles:

1. Mehdi, S.M.M., Krishnamoorthy S., Szczesniak, M.W. and Ludwików, A., 2021. "Identification of Novel miRNAs and Their Target Genes in the Response to Abscisic Acid in *Arabidopsis*" *International Journal of Molecular Sciences* 22, no. 13: 7153. https://doi.org/10.3390/ijms22137153.
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   Ministry points (MNiSW) - 140

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2. Mehdi, S.M.M., Szczesniak, M.W. and Ludwików, A., 2023. The Bro1-like domain-containing protein, AtBro1, modulates growth and abiotic stress responses in Arabidopsis. *Front. Plant Sci.* 14: 1157435.
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THESIS OUTLINE

Global food security could be jeopardized by a wide range of environmental instabilities that affect crop growth and development. Plants are constantly bombarded with harmful environmental factors like abiotic and biotic stressors during their life cycle. Climate change is leading to elevated temperatures, changes in precipitation patterns and salinity, and intensified drought and heatwaves, which all have negative effects on crop growth and productivity [1]. Such pressures on plant architecture represent a serious challenge for emergent sustainable agriculture at a time of significant growth of the world population. Plants have evolved complex processes to quickly sense and adapt to harsh climate change and these are crucial for their survival during biotic and abiotic stress [2]. Hyperosmotic stress, caused by lack of water in the environment, is a primary signal of stress in plants. Cell physiology is markedly affected by osmotic stress and ion-toxicity caused by salt. The ancillary effects of drought and salt stress conditions are complex and include oxidative stress. Oxidative stress can damage the components of cells including proteins, transcripts and membrane lipids, thereby affecting metabolic and other processes [3]. Plants respond to these effects by initiating diverse metabolic and physiological modifications, facilitated by a number of hormones, known as phytohormones, which are often specific to a certain type of stress. The most limiting factor for plant survival is drought, the response to which is mostly regulated by abscisic acid (ABA) [4,5].

ABA regulation of growth and development is important across the entire life cycle of plants, from seed germination to senescence. Plant growth and development is regulated by the core ABA signaling pathway, which, in the presence of ABA, controls plant responses via numerous effector proteins [6–8]. The pathway consists of three classes of proteins, including the ABA receptors, which are variously named pyrabactin resistance/pyrabactin resistance-like/regulatory component of the ABA receptor (encoded by the *PYR/PYL/RCAR* genes). In

addition, there are negative regulators of the pathway, the protein phosphatase 2C (*PP2C*) group A family, and positive regulators, *SNF1*-related protein kinases type 2, encoded by *SnRK2* genes. MAP kinases are activated downstream of the ABA core pathway. The ABA core signaling module controls the regulation of the *MAPKKK17* and *MAPKKK18* genes, which in turn regulate C-group MAPKs via mapkinase kinase 3. ABA-dependent responses under chronic stress conditions are probably controlled by these kinases, which contribute to the generation of robust and long-term signals [9]. MAPKKK18 is reported to interact directly with the ABI1 PP2C, which negatively regulates ABA signaling and, as the core ABA signaling module, regulates the ubiquitin-proteasome pathway [10]. *MAPKKK17* and *MAPKKK18* are induced by ABA and are considered to have redundant functions in ABA signaling [11]. ABA also affects the transcription of several microRNAs (miRNAs) in plants, which control downstream effector genes [12].

In last few decades, it has been well documented in numerous species that microRNAs (miRNAs) are critical for the regulation of plant physiology. miRNAs are single-stranded small RNAs, about 21 nucleotides long, which have crucial monitoring roles in eukaryotes. In plants, miRNA genes code for long pri-miRNA (primordial miRNA) transcripts that are transcribed by RNA polymerase II and form imperfect stem-loop secondary structures [13,14]. These transcripts are processed into approximately 70-nucleotide pre-miRNAs and subsequently duplexes of *miRNA/miRNA** by *DCL-1* (Dicer-like enzyme 1) in association with a dsRNA-binding protein, *HYL*-1. These duplexes are methylated by a dsRNA methylase encoded by *HEN1* (*HUA ENHANCER 1*), and are loaded into AGO1 (Argonaute1) [15–17], and then, with the help of an exportin homolog protein called HASTY [18,19], they are transported to the cytoplasm where they are cleaved into mature, approximately 22-nucleotide miRNAs. Mature miRNA strands form part of a multiprotein complex, the RNA-induced silencing complex (RISC). They guide the cleavage of matching target mRNAs by AGO1, which possesses RNA-

binding domains like PAZ and PIWI [19,20]. The effect of miRNAs on complementary target mRNAs is to promote their cleavage post-transcriptionally or to interfere with their translation [21]. Stresses, and plant hormones such as auxin and ABA, often regulate the expression of miRNAs, which are important regulators of development and stress responses in plants [22–24]. Identification of novel miRNAs and their target mRNAs, and the elucidation of the cellular context in which they function, remain important for understanding the role of miRNAs in gene regulation. Next-generation sequencing technologies have improved our understanding of the critical role miRNAs play under environmental stress conditions and also in plant development. However, a role for miRNAs in regulating the MAPK cascade that operates downstream of the core ABA signaling pathway has not been confirmed in Arabidopsis.

Therefore, we decided to divide my doctoral research thesis into two parts:

1. ***Identification of novel ABA-responsive miRNAs in the ABA signaling pathway and their target genes.***
2. ***Characterization of the responses of a protein, AtBro1, whose mRNA was identified as a target of one of these novel miRNAs, to various abiotic stresses in Arabidopsis.***

For the first part of my PhD research thesis, I aimed to identify novel ABA-responsive miRNAs and their target mRNAs and to investigate whether and how the ABA response in Arabidopsis involves miRNA regulation of the ABA signaling pathway. miRNAs that mediate roles in the ABA core signaling pathway were largely unknown prior to my work. Therefore, to gain insight into the role of miRNAs in the ABA core pathway and the downstream MAPK cascade, I performed small-RNA profiling of *ABI1*, *MKKK17* and *MKKK18* knockout lines and compared them to Arabidopsis wild-type (WT) Col-0 control plants. Thus, *A. thaliana* WT Col-0, *abi1td*, *mkkk17* and *mkkk18* seedlings were treated with ABA for 4 h to identify known and novel ABA-responsive miRNAs, alongside untreated

controls. Total RNA was then prepared as described in the Materials and Methods section of my research publication [25] and was sent to Macrogen Inc. (Seoul, South Korea) for small-RNA library construction and sequencing on an Illumina HiSeq 2500 system. After bioinformatics analysis, we analyzed an average of more than 8–10 million clean reads for each sample of sRNA obtained by high-throughput sequencing, and known and novel miRNAs from the control and ABA-treated libraries were identified. Small sRNA reads of 24 nucleotides were the most predominant sequence length, followed by 21 nt sRNAs. miRNA length is important for alignment with the RNA-induced silencing complex (RISC), which results in the degradation of the target mRNA or inhibition of its translation.

After normalization of the reads as 'reads per million' and analysis of differentially expressed (DE) miRNAs (selected with a false discovery rate (FDR) less than 5%), I observed that 23 of the 85 known miRNAs identified were upregulated, whereas 45 were downregulated in WT Col-0. I also identified a few mature miRNAs as novel and named them *ath-miRn-1*, *ath-miRn-2*, *ath-miRn-3*, and so on. There were ten novel miRNAs, of which six novel miRNAs were upregulated, and three downregulated after ABA treatment. In mutant seedlings of the *abi1td* genotype, three DE known miRNAs (*ath-miR824-3p*, *ath-miR2111b-3p*, *ath-miR408-5p*) were upregulated after ABA treatment. In *mkkk17*, five of seven DE known miRNAs were upregulated, two were downregulated, and one novel miRNA (*ath-miRn-8*) was highly downregulated in this mutant in response to ABA. In the *mkkk18* mutant, seven of nine DE known miRNAs were upregulated and one was downregulated. The expression levels of these miRNAs differed with genotype in ABA-induced samples, indicating that these DE miRNAs might be involved in the regulation of phase change in the presence of ABA due to its effect on post-transcriptional gene silencing.

Furthermore, I selected 15 miRNAs from the miRNA set (six known miRNAs and nine novel miRNAs) at random and performed qRT-PCR using TaqMan® miRNA assays to validate

the sequencing data. I assessed all 15 miRNAs in RNA samples from mock- and ABA-treated WT Col-0, *abi1td, mkkk17* and *mkkk18* mutants. I observed that, of the six known miRNAs, after ABA treatment, three were upregulated and three downregulated across all genotypes, while three of the novel miRNAs were downregulated and the remaining six were upregulated. Thus, qRT-PCR data for *ath-miR2111b-3p, ath-miR824-3p, ath-miR171-5p, ath-miR472-3p, ath-miRn-4, ath-miRn-6* and *ath-miRn-8* broadly matched the NGS data in almost all genotypes. However, I found that some miRNAs showed differences between the qRT-PCR and NGS data: for example, *ath-miR846-3p, ath-miRn-1, ath-miRn-2, ath-miRn-3, ath-miRn-5, ath-miRn-7* and *ath-miRn-9* were either upregulated after ABA treatment according to the sequencing data, but downregulated according to the qPCR data, or vice versa. qPCR analysis indicated that, although the fold change in expression did not completely coincide with that indicated by the sequencing data, the trend was similar, as shown in a previous study [26]. My results indicate the reliability of high-throughput sequencing as a method of predicting miRNA expression profiles.

My next task was to identify the target genes for both the known and validated novel miRNAs using a web-based database, psRNATarget. To test these predictions, I used 5′ RLM-RACE to validate the miRNA cleavage sites in target mRNAs. This method maps the 5'-ends of target mRNAs within the expected cleavage site by taking advantage of the characteristics of AGO-mediated cleavage. This cleavage leaves ligation-competent 3'-cleavage fragments ending with 5′-monophosphates. In a modified protocol, particular steps (dephosphorylation and cap-removal reactions) from the original 5'-RACE protocol were skipped. A 5'-RNA adaptor was directly ligated to the 3'-end of miRNA-directed cleavage products that contain a ligation-competent 5'-monophosphate [27]. The ligated RNAs were reverse-transcribed, PCR-amplified, cloned and sequenced, allowing determination of their mRNA cleavage sites. Indeed, I confirmed cleavage sites for six of the novel miRNAs in seven target genes, which supports

the view that these genes might be direct targets of the corresponding miRNAs in Arabidopsis. These findings verified cleavage sites in target genes *AT1G73390* (*AtBro1*, which encodes a Bro1-like domain containing protein) for *ath-miRn-1*, *AT5G40550* (*SGF29b*) for *ath-miRn-2*, *AT3G14070* (*CAX9*) for *ath-miRn-4*, *AT1G56650* (*MYB75*), *AT5G58490* for *ath-miRn-6*, *AT3G15570* (phototropic-responsive NPH3 family protein) for *ath-miRn-8*, and *AT2G29140* (*PUM3*) for *ath-miRn-9*. To test whether novel miRNA target genes are regulated by ABA, I performed qPCR on selected novel miRNA-targeted genes (such as *AT1G73390*, *AT5G40550*, *AT3G15570* and *AT5G58490*) that showed positive ABA-responsive expression in Arabidopsis WT Col-0, *abi1td*, *mkkk17* and *mkkk18* mutants before or after 4 h ABA treatment. My results show that these selected miRNA target genes were significantly regulated by ABA in almost all genotypes.

Plant growth and development are regulated by complex gene networks. Target gene identification and, subsequently, GO analysis and KEGG pathway assignment linked our panel of miRNAs and their target genes with various biological processes, cellular components and molecular functions. Interestingly, the top-10 enriched roles of the target genes of up- and downregulated miRNAs, i.e., the molecular processes ‘ion binding’, ‘protein binding’, ‘ATP binding’, ‘nucleotide binding’, ‘metal ion binding’, ‘DNA binding, ‘transferase activity’, ‘nucleic acid binding transcription factor activity’ and ‘kinase activity’, were much more enriched in WT Col-0 than in the *abi1td*, *mkkk17* and *mkkk18* mutants after ABA treatment. All biological and molecular processes, including signaling, response to stimulus, developmental process, and reproductive process, were found to be regulated by the target genes of DE miRNAs after ABA treatment in all genotypes, which suggests that these miRNAs in Arabidopsis have a broad regulatory role in ABA signaling.

These results are important for further research on the role of the target genes of the novel miRNAs described above. All the results of the first part of my PhD thesis were published in

the article: Mehdi, S.M.M.; Krishnamoorthy, S.; Szczesniak, M.W.; Ludwików, A., 2021. **"Identification of Novel miRNAs and Their Target Genes in the Response to Abscisic Acid in *Arabidopsis*"** *International Journal of Molecular Sciences* 22, no. 13: 7153.

For the second part of my PhD research thesis, I investigated *AT1G73390* (*AtBro1*), a potential target gene of one of the novel miRNAs identified, *ath-miRn-1,* whose expression was suppressed by ABA in WT along with other genotypes [25]. Bro1 is a protein domain in which the N-terminal half resembles a boomerang or banana shape [28] and is involved in a number of different processes. First, in yeast, the viability of *bro1* cells is reduced during nutrient starvation, resulting in defective regulation of cell proliferation [29]. In addition, Doa4, a ubiquitin thiolesterase that regulates membrane scission in endosomes, requires Bro1 for its recruitment to endosomal membranes [30,31]. Multivascular body (MVB) formation during phagocytosis is mediated by EhADH112, a Bro1-domain protein, on the cell surface and in endosomal compartments in anaerobic parasitic amoebozoans [32,33]. *AtBro1* (*AT1G73390*) is one of only five genes in the Arabidopsis genome that encode Bro1-domain proteins, the others being *BRAF, AT1G17940, ALIX* and *AT1G13310* [34]. The function of the AtBro1 protein was totally unknown and uncharacterized prior to my work. Therefore, I decided to focus on the role of the AtBro1 gene in response to different abiotic stress factors. AtBro1 is a protein of 419 amino acids with one Bro1-like domain. Using *BLASTP*, I identified one paralog (*AT1G17940*) and numerous orthologs in other plant species.

Next, I used qRT-PCR to assess transcript levels in WT seedlings and found *AtBro1* to be significantly upregulated by the phytohormone ABA, and also by salt and mannitol stress. During WT seedling development, *At1Bro1* transcript levels were high, reaching a peak after 21 days. *AtBro1* was expressed in various tissues, including stems, leaves, roots, cotyledons, apical buds, flower buds and flowers under normal conditions and at different developmental stages, but transcript levels were higher in leaves and flowers than in roots and siliques. I also

showed that the increase in transcript levels in flower clusters and rosette leaves was more abundant than in other tissues of Arabidopsis plants.

I analyzed the promoter sequence of *AtBro1*, in which core and *cis*-regulatory elements were identified using a plant *cis*-acting regulatory elements database. The promoter contained several stretches of *TATA* box elements and, at discrete locations, various *CAAT* boxes, along with other *cis*-regulatory elements such as *ABRE4*, *GARE* and *Myb* motifs. Subsequently, transgenic plants were produced that expressed a pAtBro1::β-glucuronidase (GUS) construct. GUS expression was apparent at all stages of plant development but was higher in floral tissues than in other tissues, especially in anthers; GUS was also expressed in cotyledon leaves. As found in leaves, the low level of GUS staining in seedlings and at the flowering stage reflected the results obtained by qRT-PCR. Because *AtBro1* was strongly induced by ABA, I treated two-week-old seedlings of transgenic pAtBro1::GUS plants with ABA and observed strong GUS expression in the leaves, but no expression in roots. This is similar to the expression pattern of some AtβCA family genes, such as *AtβCA2p*, whose transcripts are completely absent in roots, but accumulate to high levels in floral clusters [35]. *AtβCA2p* is also reported to be involved in stress responses [36]. The *AtSRP2* and *AtSRP3* genes behave similarly: they show marked expression in floral clusters, but not in roots [37], and are involved in the response to stress [38]. The above analysis suggests that AtBro1 might be involved in both vegetative and reproductive development in Arabidopsis.

To verify the subcellular localization of AtBro1, a C-terminal fusion of AtBro1 with green fluorescent protein (*GFP*) was expressed in Arabidopsis protoplasts. AtBro1-GFP fusion proteins were found exclusively in protoplast membranes. This was confirmed using the lipophilic dye, FM4-64, which incorporates into the outer leaflet of plasma membranes where it emits an intense fluorescence between 580 nm and 650 nm. The protoplasts were subsequently stained, using a final concentration of 0.5% (*v/v*) of the FM4-64 stock solution (1

μg/μl). After an incubation period of 10-15 min at room temperature, FM4-64 stained all areas of the plasma membrane and colocalized with AtBro1-GFP [39]. These results suggest that AtBro1 is involved in the regulation of plasma membrane homeostasis in Arabidopsis cells.

I then investigated the role of AtBro1 in the plant response to abiotic stress by physiological, biochemical and genetic characterization of the mutant line, *bro1* (Salk line with T-DNA insertion), along with AtBro1-overexpression lines (*OX 7-5, OX 11-4* and *OX 16-5*). To confirm the function of AtBro1 in stress responses of the *bro1* mutant, I also produced complementary lines (*Comp-1* and *2*) obtained by crossing the *bro1* knockout plant with an OX transgenic line showing similar AtBro1 expression levels to WT plants, as described in my research publication [40]. My experiments focused on gaining a better understanding of AtBro1 function during abiotic stress. To elucidate the role of AtBro1 in stress responses, I used all the above-mentioned lines, which were grown with or without ABA or mannitol. I noticed that the *bro1* mutant showed less sensitivity than WT during seed germination on medium with ABA or mannitol, whereas the OX transgenic lines were more sensitive to the treatments. In response to ABA and mannitol treatment, the germination rate was reduced in OX-line seeds compared to WT, although the *bro1* mutant showed more resistance to ABA inhibition than WT. I also showed that the phenotypes of the complementary lines were almost identical to those of WT seedlings under ABA and mannitol stress conditions. These results led me to conclude that AtBro1 is positively involved in the response to ABA and mannitol during germination.

To better understand the observed abiotic stress response, I also tested whether AtBro1 modulates the effect of salt stress on seed germination. The germination of *bro1* mutant seeds was inhibited in the presence of NaCl compared to WT, but the germination rates and cotyledon greening ratios were much higher in OX transgenic lines. The *bro1* phenotype was restored to that of the WT in complementary lines, however. I also performed a salt-stress growth assay

to further investigate the function of AtBro1 in NaCl tolerance. I transferred seedlings to medium with different salt concentrations or without salt and allowed them to grow vertically. I observed that the *bro1* mutant showed a hypersensitive phenotype compared to WT Col-0, whereas the OX lines were more resistant than WT. Similarly, after salt stress treatment, the fresh weight of *bro1* mutant seedlings was significantly reduced compared to WT, while the fresh weight of the OX transgenic lines was significantly higher. These results indicate that AtBro1 is negatively involved in the response to salt stress during seed germination and seedling growth. However, in the drought assay, the *bro1* mutant was more sensitive to drought, as shown in my research publication [40], whereas the OX lines were more drought-resistant than WT. Interestingly, I noticed that the highest survival rate after re-watering occurred in OX lines, i.e. higher than both WT and the *bro1* mutant. Electrolyte leakage is a biological marker of cell-membrane damage in plants [41–44]. Exposure to drought caused phenotypic differences and more severe electrolyte leakage in the *bro1* mutant than in OX lines or WT plants. OX lines showed less wilting, which was associated with lower levels of electrolyte leakage, revealing that *AtBro1* overexpression improved the survival rate of transgenic lines compared to WT and the *bro1* mutant.

Together, these observations showed that *AtBro1*-OX plants were hypersensitive to mannitol stress during seed germination but had greater drought resistance than WT and *bro1-1* mutant plants. Thus, it appears that *AtBro1* may be involved in ABA-regulated responses rather than the response to osmotic stress. This is similar to *AtAIRP4*, as *AtAIRP4*-OX plants are hypersensitive to osmotic stress during seed germination, but more resistant than WT to drought stress [45]. Several stress-related genes, including *SnRK2.3*, *NCED3*, *AAO3*, and *RD29B*, were more highly expressed in *AtBro1*-OX plants than in WT Col-0, whereas *ABA1* and *RD29A* expression was lower in the OX lines. Such changes in transcript expression are indicative of the degree of stress sensitivity or stress tolerance under harsh conditions [46–48].

Next, I was interested to study the role of *AtBro1* at the level of transcriptional regulation. RNA-sequencing was performed to investigate the possible biological effects of *AtBro1* activity and to identify the associated regulatory network of genes involved in plant growth and the response to various abiotic stresses. I performed next-generation mRNA-sequencing to explore the set of genes that were differentially expressed (DEGs) between two-week-old WT and *bro1* mutant seedlings after treatment with ABA for 0, 2 and 4 h. Various abiotic stress-responsive genes were found to be repressed or induced according to the RNA-seq data, including *MOP9.5*, *MRD1*, *HEI10*, and *MIOX4* [49–52], which have previously been shown to be regulated by abiotic stress or ABA. Moreover, *AtBro1* also influenced the expression of several genes associated with other Arabidopsis processes. Transcriptome and Gene Ontology analysis of *bro1* versus WT Col-0 with or without ABA treatment revealed that *AtBro1* affects the transcriptional regulation of genes involved in various biological functions, especially cellular response to ABA stimulus, response to biotic stimulus, and regulation of response to stress, showing that *AtBro1* might have an important function in the response to abiotic stresses and in hormone signalling. The analysis showed that many genes were significantly enriched in terms related to 'external stimulus', 'response to hormone', 'defense response' and 'hormone-mediated signaling pathway', indicating that the enhanced seed germination of the *bro1* mutant may be due to increased 'ion and osmotic signaling', which is involved in the induction of downstream stress-responsive genes, including genes that respond to ABA. I also used qRT-PCR to validate some of the RNA-seq data, showing that the expression of *PI4KGAMMA3/MOP9.5*, *MRD1* and *HEI10* was lower in *bro1* mutant plants than WT, but higher in OX lines during ABA treatment. Therefore, I concluded that the role of *AtBro1* in abiotic stress tolerance might be to regulate ABA-responsive genes at the transcriptomic level.

The above results are crucial for an improved understanding of the involvement of AtBro1 protein in balancing plant stress response and growth and show that AtBro1 is a promising

candidate for interventions that might improve plant stress resistance. All the results of the second part of my doctoral thesis were recently published in the article: Mehdi, S.M.M.; Szczesniak, M.W.; Ludwików, A., 2023. ***The Bro1-like domain-containing protein, AtBro1, modulates growth and abiotic stress responses in Arabidopsis***. ***Front. Plant Sci. 14: 1157435***.

SUMMARY

To summarize collectively, in my PhD thesis, I identified 10 novel miRNAs in WT Arabidopsis, most of which were regulated by ABA, while in *abi1td*, *mkkk17* and *mkkk18* mutants, three, seven and nine known miRNAs, respectively, were differentially expressed after ABA treatment. In response to ABA, one novel miRNA (*ath-miRn-8*) was differentially expressed in the *mkkk17* mutant. Using bioinformatics, I identified mRNA targets of these novel miRNAs; seven target genes of six novel miRNAs were further validated by *5′ RLM-RACE*. Gene Ontology analyses predicted these potential target genes to be involved in diverse cellular processes in plants, including development and stomatal movement. It will be interesting to investigate the role of the remaining ABA-responsive novel miRNAs that we reported here, together with their putative mRNA targets, in plants responding to environmental challenges during plant growth and development.

I also characterized the *AtBro1* gene, which was identified as a target of one of the novel miRNAs. I identified AtBro1 as a plasma membrane-localized protein and a crucial regulator involved in responding to abiotic stress conditions when environmental stress factors are absent or at low levels. From our experimental data, AtBro1 could also be considered to be involved in the regulation of plant stress tolerance by increasing or decreasing the expression of a large number of stress-responsive target genes, including *MOP9.5*, *MRD1*, *HEI10* and *MIOX4*, to maintain ion homeostasis and elicit the critical response to various stress factors. Lastly, my study suggests that AtBro1 may be involved in balancing plant stress response and growth, and thus represents a promising candidate for interventions that improve plant stress resistance. Moreover, in future, it will be interesting to study the relationship of *MOP9.5*, *MRD1*, *HEI10* and *MIOX4* with AtBro1 protein during abiotic stress responses in Arabidopsis.

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PUBLICATION #1

Identification of Novel miRNAs and Their Target Genes in the Response to Abscisic Acid in Arabidopsis

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My total contribution to the work was 75%.

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My total contribution to the work is 5%.

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PUBLICATION #2

The Bro1-like domain-containing protein, AtBro1, modulates growth and abiotic stress responses in Arabidopsis

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(Supplementary data files are available in online Open Access Source of research article journal)

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Statement of contribution to the research article

I hereby declare that my contribution to the article: ***Mehdi, S.M.M.; Szczesniak, M.W.; Ludwików. 2023. The Bro1-like domain-containing protein, AtBro1, modulates growth and abiotic stress responses in Arabidopsis. Front. Plant Sci., 4: 1157435,*** co-designed the study, performed all experiments for research article, analyzed the data from qRT-PCRs and other experiments, conducted statistical analysis, prepared figures and tables for publication, interpreted the data, visualization of data, analysis of data from deep sequencing, discussion of the obtained results, written and drafted the manuscript, performed review of manuscript and approval of final version of manuscript to be published, correction of the manuscript after reviewers' ratings.

My total contribution to the work was 80%.

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Statement of contribution to the research article

I hereby declare that my contribution to the article: ***Mehdi, S.M.M.; Szczesniak, M.W.; Ludwików. 2023. AtBRO1, BRO1-like domain-containing protein, modulates growth and abiotic stress responses in Arabidopsis. Front. Plant Sci. 14: 1157435,*** was involved in small RNA data analysis from deep sequencing, performed review of manuscript and approval of final version of manuscript to be published.

My total contribution to the work was 5%.

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I hereby declare that my contribution to the article: ***Mehdi, S.M.M.; Szczesniak, M.W.; Ludwików. 2023. The Bro1-like domain-containing protein, AtBro1, modulates growth and abiotic stress responses in Arabidopsis. Front. Plant Sci. 14: 1157435,*** co-designed the study, supervised the study, discussion of the obtained results, performed review of manuscript and approval of final version of manuscript to be published, correction of the manuscript after reviewers' ratings, organized funding for the research through the NCN project.

My total contribution to the work was 15%.

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