DIPLOMA THESIS

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BUDAPEST

Functional Analysis of *ago1*, *ago2*, and *ago10* mutants

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ABBREVIATIONS

AGO	: Argonaute
ATP	: Adenosine Three-Phosphate
DCLs	: Dicer-like RNase III endonucleases
DNA	: Deoxyribonucleic Acid
dsRNA	: double stranded RNA
HD-ZIPIII	: *HOMEDOMAIN-LEUCINE ZIPPER III*
HEN1	: HUA Enhancer 1
HYL1	: HYPONASTIC LEAVES 1
miRNA	: microRNA
PHB	: *PHABULOSA*
PHV	: *PHAVOLUTA*
pre-miRNA	: precursor-miRNAs/
pri-miRNAs	: primary miRNA
PTGS	: posttranscriptional gene silencing
RAPD	: Random Amplified Polymorphic DNA
RFLP	: Restriction Fragment Length Polymorphism
RISC	: RNA induced gene silencing complexes
RNA	: Ribonucleic Acid
RNAi	: RNA interference
SAM	: Shoot Apical Meristem
SDN	: SMALL RNA DEGRADING NUCLEASES
SE	: SERRATE
siRNA	: small interference RNA
SPL	: *SQUAMOSA promoter-binding protein-like*
TGS	: Transcriptional gene silencing

1. INTRODUCTION

ARGONAUTEs (AGOs) form a protein group that exists in eukaryotic and procaryotic cells having a function in gene silencing pathways together with small, 21-24 nt long RNAs (sRNA) as the guide. Based on sequence complementarity with the given small RNA, AGOs target and silence the DNA or RNA by directly cutting the targeted strand or blocking the expression. In other terms, AGO – sRNA structure is involved in two recognizable gene-silencing pathways: Transcriptional gene silencing (TGS) where in some cases, the sRNAs target specific promoter regions and promote methylation activity, and posttranscriptional gene silencing (PTGS) which involves either the degradation or transcriptional inhibition of targeted mRNA.

AGOs loaded with sRNAs together with several other protein co-factors form rather large RNA-induced gene silencing complexes (RISC), which are the effectors of the pathway and due to the endonuclease activity of some AGOs, can trigger cutting of the target RNA. sRNAs play a major role in guiding AGOs into specific targets through partial or full sequence complementarity. In this regard, there are multiple types of sRNAs. However, from the developmental point of view, one of the most important sRNA groups is the microRNA (miRNA) group. MiRNAs are 20-24 nucleotide long noncoding RNAs expressed from MIR genes both in plants and animals targeting mostly endogenous mRNAs, particularly several transcriptional factors.

According to *Arabidopsis thaliana* sequence data, the 10 AGO proteins usually inherent in every plant were rendered into three major clades based on phylogenetic sequence analysis: AGO4/6/8/9, AGO2/3/7, and AGO1/5/10 with additional AGO18 in monocots belonging into the latest group. As this clustering is based not on functionality, AGOs belonging to different groups can share similar roles and vice versa closely related proteins can have partially distinct functions, as well. As an example, it has been shown that AGO1 and AGO7 give rise to proper leaf development while AGO3 and 4 promote DNA methylation, although those two AGOs are in different clades, phylogenetically. All of those discoveries are mostly about single AGO functional studies. The way how AGOs collaborate in the regulation of the development and reproduction of plants is still poorly understood. Indeed, the function of a single AGO protein on the development of the plant can be studied by suppressing or disabling the gene that expresses the particular AGO protein and observing the alterations in the development and function that may occur in the mutant plant. It has been studied that *ago1-27* and *ago1-25* single mutants show phenotypical defects as dark, serrated leaves and low fertility (Morel *et al.,* 2002). However, the study to understand the possible redundant functions of other AGOs in terms of development and their role in controlling certain mRNAs can be conducted only in an *ago1* mutant background.

Thus, this study focuses on revealing the function of AGO1, AGO2, and AGO10 in the development of plants (*Arabidopsis thaliana*) with the help of single, double, and triple mutants inactivated in the respective AGO genes. As it was previously described, AGO1 protein can protect certain miRNAs and in the lack of AGO1, these can accumulate to a lesser extent. To monitor whether other AGOs can share the same task in *Arabidopsis,* miRNA abundance analysis can be carried out. Although with a lower correlation due to the difficult and multi-layered regulation, the abundance of certain miRNA target mRNAs can also be informative. And finally, the number of active RISCs is possible to be monitored with the gel-filtration method.

This study was conducted in the Plant Developmental Biology Group of the Hungarian University of Agriculture and Life Sciences (MATE-GBI) which has already been working with miRNAs for several years.

2. OBJECTIVE

The objective of this research is to analyze the functional redundancy of AGO1, AGO2, and AGO10 1) through phenotypical analysis of single, double, and triple mutants impaired in the respective genes. 2) To investigate the possible substitution or backup function of AGO2 and AGO10 in the miRNA pathway with the analysis of miR168 and miR159 content in the mutant plants. 3)To investigate the expression of AGO1 mRNA as the target of miR168 in the presence of analyzed mutations, and 4) to visualize the loading efficiency of miR168 into AGO-RISC complex in *ago1-25* and *ago1-25, 2-1, 10-1* mutants.

3. LITERATURE REVIEW

3.1 RNA interference

Numerous experiments from the 1990s revealed some interesting phenomena of silencing, the expression of different exogenous genes producing double-stranded or small single-stranded RNAs. These results led researchers to the identification of the mechanism of gene silencing. **(Fig. 1).** One of these findings was an experiment on Petunia to over-express Chalcone synthase, a crucial enzyme in flavonoid or isoflavonoid biosynthesis pathway prove to be a blocking agent in Anthocyanin biosynthesis, resulting in white flowers on forty-two percent of the offspring (Napoli et al*,* 1990). Several years later, another experiment showed that the phytoene desaturase (PDS) gene was silenced in *Nicotiana benthamiana* when the plant was infected with potato virus X vectors containing the same gene. The same phenomenon was observed with the green fluorescent protein transgene. These were explained with the theory of the introduced mRNA in a virus vector which resulted in a double-stranded RNA and triggered RNA-induced gene silencing (Teresa et al. 1998).

In the same year, RNA interference (RNAi) was discovered in an experiment where a single-stranded short RNA (ssRNA) and a double-stranded RNA (dsRNA) with the same complementary strand were introduced in *Caenorhabditis elegans.* A phenotypic observation showed a more severe outcome on the introduction of double-stranded RNA, resulting in albino individuals and offspring due to the degradation of RNA strands both sense and antisense (Fire et al. 1998). Thus, RNAi is defined as a gene silencing mechanism that is conserved in plants and animals as well, and mediated by the small RNAs (sRNA). Nevertheless, the observation regarding gene silencing and its applications keeps going these days, including one of the most crucial discoveries, the CRISPR system which is also an important gene silencing mechanism in bacteria and led to an efficient genetic engineering tool in eukaryotes (Mojica et al. 2005).

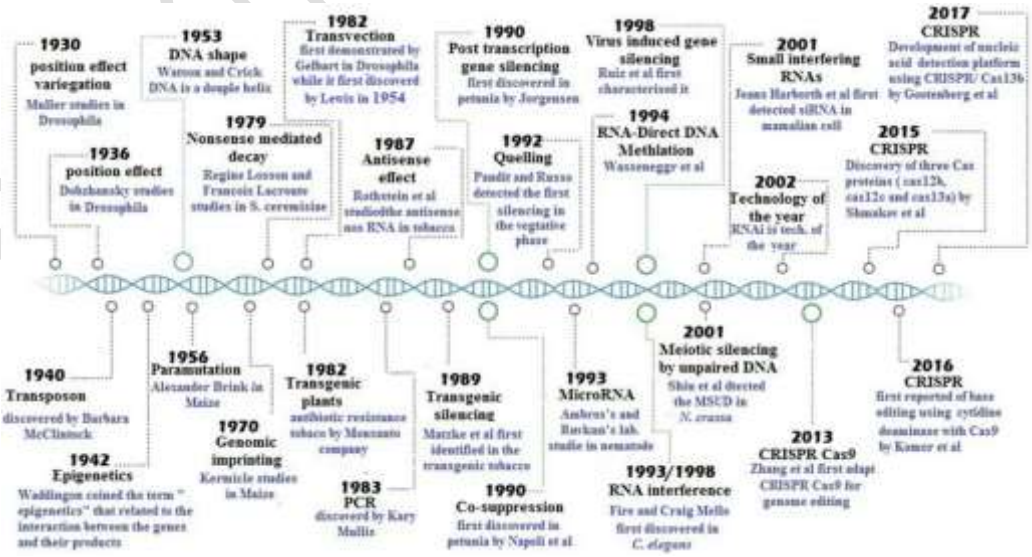

Figure 1. History of gene silencing (El-Sappah et al. 2021)

Transcriptional gene silencing (TGS) is a process that modifies gene expression without changing the DNA sequence, an epigenetic modification that is heritable in most cases and leads to the inactivation of previously active genes. During normal development and differentiation of the cells in an individual, some genes are not needed to be expressed, thus the cells begin to differentiate into some specific functions, and silencing mechanisms prevent unnecessary genes to be expressed and producing unwanted products (Filipowicz et al. 2013).

3.2 Small RNAs

3.2.1 Definition and Differentiation

As a part of the RNA interference silencing mechanism, it has been studied that small RNA pathways were an evolutionary adjustment, emerging from the original form of the defense mechanism of cells against transposable elements and RNA viruses to an endogenous gene expression regulatory system. Based on their biogenesis and origin, small RNAs are divided into two main groups, microRNAs (miRNA), and small interference RNAs (siRNA) while their function can overlap. miRNAs originate from long single-stranded RNAs expressed from endogenous MIR genes; they are folded to create a hairpin-like structure with an imperfect pairing of a double-stranded RNA (dsRNAs) section. This primary miRNA (pri-miRNA) structure is processed by RNase III proteins (Drosha or Dicer family) to become mature miRNA. Generally, there is only one miRNA produced from a *MIR* gene. On the other hand, siRNAs are produced from long perfect dsRNAs originated from inverted repeat sequences or transcription of sense-antisense gene pairs and can not only regulate unrelated origin of transcribed sequence but also the gene or sequence from which the siRNA originated (Mallory et al. 2006). The siRNA precursors usually produce several small RNAs.

In plants, there are multiple ways of small RNA biogenesis and these can be majorly divided based on their involvement in post-transcriptional gene silencing (PTGS) including mRNA cleavage and translational repression, and/or transcriptional gene silencing (TGS) **(Fig. 2).** While miRNAs are the contributors to the degradation of mRNAs in a sequence-specific manner or translational repression on the first step of translation initiation, secondary siRNAs can contribute also to DNA methylation together with heterochromatic siRNAs (Borges and Martienssen, 2015).

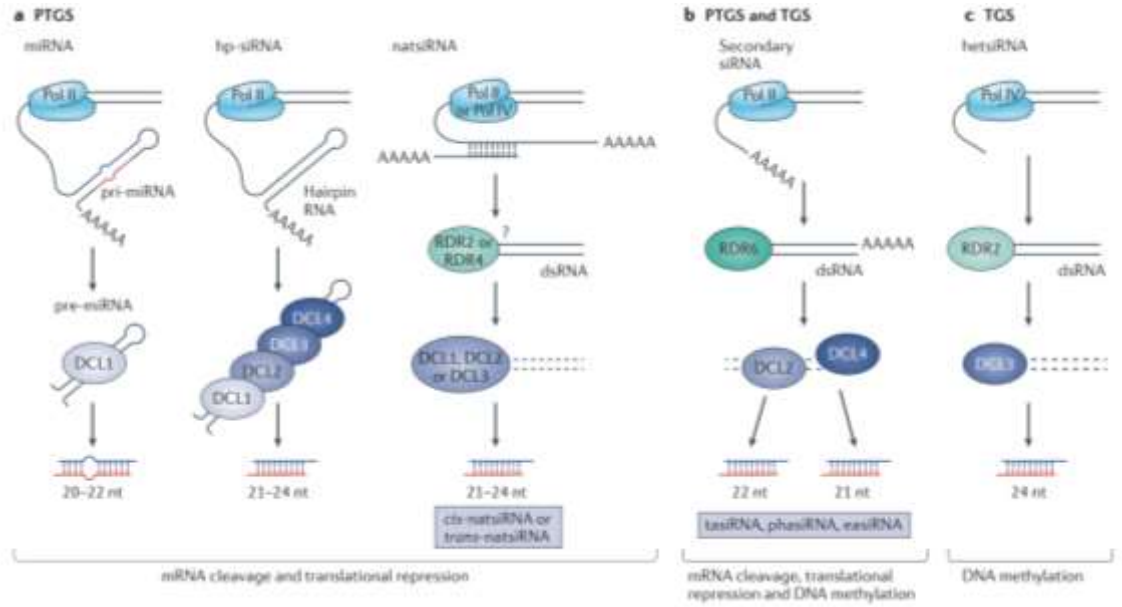

Figure 2. Biogenesis of different types of small RNAs (Borges and Martienssen, 2015)

DICER-LIKE proteins (DCL) play a major role in small RNA biosynthesis (**Fig. 2**). DCL proteins recognize the presence of dsRNA which was produced from either endogenous gene, any pathogen, or transgene or by the help of RNA-DEPENDENT RNA POLYMERASES (RDRs). DICER proteins in every eucaryote possess a slicing activity and help the precursor small RNA to become a mature small RNA. Though some types of DCLs can be contributed to the same or different types of small RNA, microRNAs are mostly exclusively sliced by DCL1.

3.2.2. The microRNA pathway

MicroRNAs (miRNAs) as one the of small RNA classes were identified for the first time in 1993. A large number of regulatory activities in animals, expression patterns, ancient evolutionary identity, and generality of their activities were recorded in the following decade. In plants, enormous studies have found their roles in several aspects including growth, development, and plant response to external factors (Shriram et al. 2016; Li et al. 2017; Brant et al. 2018).

The origins of miRNAs are polymerase II transcribed endogenous genes called MIR genes. There are more than 100 MIR in the majority of plants with specific transcriptional units. During transcriptional initiation, a mediator protein is interacted with transcriptional activators and leads to Pol II recruitments with possible trans-acting factors affecting the transcription due to the existence of many cis-regulatory sequences in the promoter element. The existence of some transcription factor (TF) can contribute a major influence on the transcription of the MIR gene other than the TATA box core. Some TFs could act differently to some MIR genes, whether to suppress or to enhance while some miRNAs could also target some TF mRNAs to silence its strand and thus affect the expression of other MIR genes (negative feedback). For example, APETALA2 (AP2) TF could increase MIR156 gene expression but act antagonistically to the MIR172b gene. Another TF such as SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) acts similarly to AP2. Some SPL

proteins are a silencing target of miR156. The elevation of this SPL due to the lower abundance of miR156 could lead to higher efficiency for MIR172 to be expressed due to the elevation of SPL TF (Song et al. 2019).

Furthermore, the 3' polyadenylate tail and 5' 7-methylguanosine cap are added to the primary miRNA (pri-miRNA) from those transcribed *MIRs* to stabilize the sequence. Then a stem-loop shape is formed from the single pri-miRNA based on the partial sequence complementary of the pri-miRNA parts. It is important to note that pri-miRNAs can possess introns. Thus, the presence of introns leads to alternative splicing affecting the formation of different stem-loop structures and the final amount of produced miRNA. The Dicer-like RNase III endonucleases (DCLs) such as DCL1 and DCL4 in *Arabidopsis thaliana*, recognize the folded-up hairpin-like stem-loop pri-miRNA structures, discharge the stem and loop part (precursor-miRNAs/pre-miRNA) and following this, generate the miRNA/miRNA* duplex. DCL1 have co-factors in this process like HYPONASTIC LEAVES 1 (HYL1) and SERRATE (SE), and together they form the dicing complex. The duplex is a small double-stranded RNA (guide and passenger strands together) and contains a 2 nucleotide (nt) long 3' overhang. The hydroxyl groups, particularly 2'OH at the end of each strand are methylated by HUA Enhancer 1 (HEN1) to protect the duplex from degradation and uridylation. HST1 protein then transports the duplex into the cytoplasm. Finally, only a single strand from the duplex is loaded into the AGO1 in an RNA-induced silencing complex (RISC) (**Fig.3** Left) (Mica et al. 2009; Ren et al. 2012; Wang et al. 2019).

However, a recent study suggested that in plants, all the maturation, methylation, loading activity of mature miRNA into Argonaute (AGO) protein, and discharging of unused RNA strands are occurring in the nucleus. And AGO-mature miRNA complex is transported together to the cytosol and can also possibly be transported back to the nucleus in the NES (Nuclear-export signal)-dependent manner which exists in the N-termini of AGO1 protein (**Fig. 3** Right). Thus, AGO1 is shuttling and transporting mature miRNA between the nucleus and cytosol (Nicholas et al. 2018).

Furthermore, it was observed recently that a great amount of miRNA is not loaded directly into RISCs and exists freely in the cytosol, creating an unbound pool. Additionally, to this observation, it was also reported that individual miRNAs have various tendencies to be loaded and consequently produce different amounts of the free pool (Dalmadi et al. 2019). The loading efficiency of the different miRNAs depends on either the relation of free AGO1 available in the cell and the number of competitors' sRNAs (Dalmadi et al. 2019) or the structural features of the pre-miRNA (Dalmadi et al. 2021).

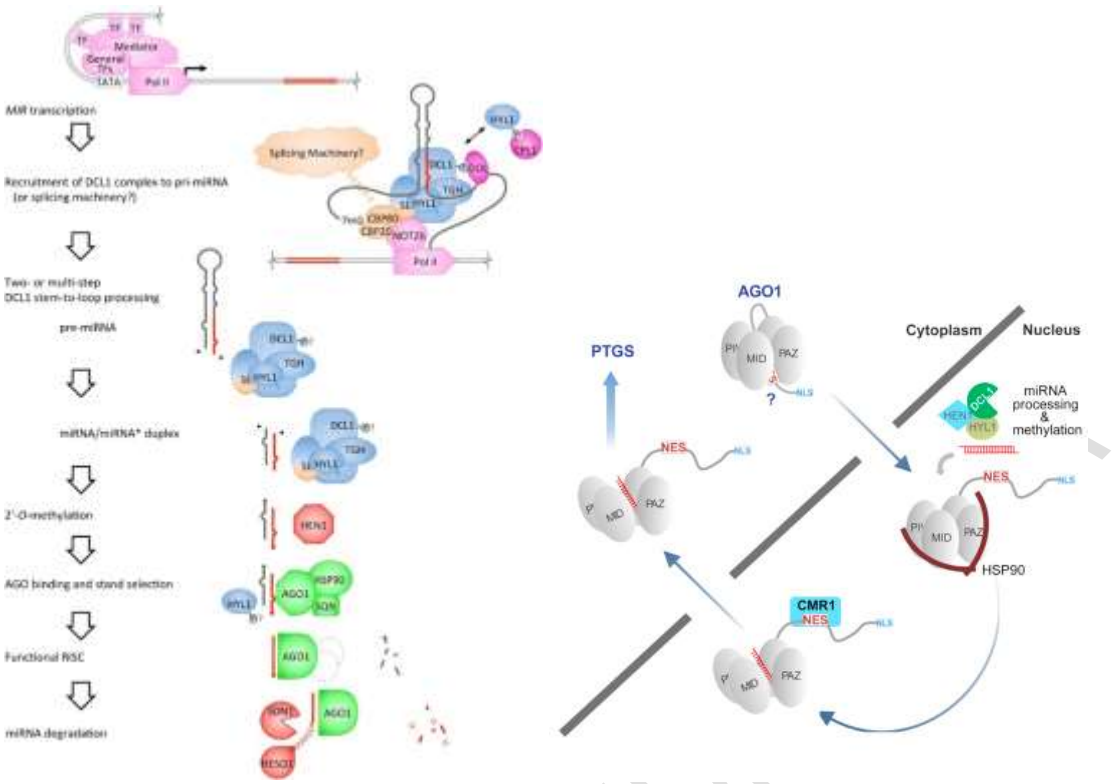

Figure 3. Biogenesis, action, and degradation of microRNAs (left) and NES-dependent manner of miRNA-AGO translocation (right) (Rogers and Chen, 2013; Nicholas et al. 2018).

3.2.3 miRNAs related to current work

Some important miRNAs have outstanding roles in plant development. Mir168 is a key component in the control of the whole miRNA pathway through the regulation of ARGONAUTE1 mRNA itself in a negative feedback manner. As AGO1 is one of the most important AGO proteins necessary for plant development that is expressed for the entire life of the plant, miR168 action is also important. Additionally, mR159 is shown to play an important mission in the development of plant and seed germination and the response to the drought by controlling the activation of the ABI5 protein which suppresses miR156. Mir156 focuses on the transition of plant phase development changes by suppressing the protein responsible for leaf development during the vegetative phase. GAMYB transcription factor which binds to the TAACAA box of specific gene promoters (in this case LEAFY gene) to be transcripted becomes the target of this miRNA to ensure the normal growth of the plant. Thus, miR159 could affect miR156 function which then leads to the development of leaf organs during the vegetative phase. Moreover, under abiotic stress, the ABA that accumulated from this event can elevate the mir159 expression (Gubler et al. 1999; Wu et al. 2006; Vaucheret, 2006; Liu et al. 2017; Jiang et al. 2022). The two miRNAs (miR168 and miR159) involved in the current investigation were also selected by their differing nature in terms of loading capacity. As reported before, while miR168 is rather poorly, miR159 is highly loaded into RISC (Dalmadi et al. 2019). According to this, they can have slightly different behavior in the investigated mutant lines.

3.3 Argonaute (AGO) Proteins

AGO proteins guided by the different types of sRNAs are the key effectors of the RNA interference pathways and form RNA-induced silencing complexes (RISCs). These nucleo-protein complexes can either evoke DNA methylation through transcriptional gene silencing (TGS) or target RNA through the posttranscriptional gene silencing (PTGS) mechanism. There are four functional domains in every AGOs: PIWI, MID, PAZ, and diverse N-domain with two linker regions, L1 and L2. MID binds the 5' mono-phosphorylated nucleotide while PAZ binds the 3' nucleotide of sRNA. Furthermore, the PIWI domain has a slicer activity due to the existence of metal-coordinating residues and thus, can cleave the target mRNA. It has been proven that AGO1, AGO2, AGO4, AGO7, and AGO10 of *Arabidopsis thaliana* have slicing activity (Carbonell, 2017).

In general, there are 10 AGO proteins in *Arabidopsis thaliana,* grouped into 3 clades phylogenetically; AGO1/5/10, AGO2/3/7, and AGO4/6/8/9 (**Fig. 4**). Some suggested that this diversity exists due to genome duplication that happens often in plant species to provide some specificity among plant cells and organs and to facilitate individuals' ability to respond to specific conditions (Han et al. 2015). Consequently, the biological roles of each AGOs can partially be distinct and overlapping ensuring the homeostasis of essential functions with redundancy. For example, it has been recorded in *Arabidopsis thaliana* that AGO1, 2, 4, 5, and 7 play an important role in antiviral defense, and AGO2 and 4 are also taking part in antibacterial immunity.

AGO1 has a major influence on plant development in general. Besides its inevitably essential role being the effector of the miRNA pathway, and so regulating a set of important transcriptional factors and other proteins, it participates also in siRNA-mediated pathways. Furthermore, AGO1 also contributes to stress response and together with AGO7 to leaf development while cell specification is controlled by AGO9. It has been also mentioned in a study that development is purposely regulated and maintained by AGO10 which turned out to have a higher binding affinity to miR166 than AGO1, one of the requirements for SAM development. However, there is no developmental defect of the plant recorded under the low expression of AGO10, a redundancy between AGO1 and AGO10 can be seen in the embryo development. Abnormal embryo development was detected in *ago1-1, 10-1* double mutant while this defect did not appear in each of the single mutants (Morell et al. 2002; Mallory et al. 2009; Zhu et al. 2011; Harvey et al. 2011; Carbonell, 2017).

Moreover, the *ago2-1* mutant does not exhibit any visible phenotypical changes in its physiological state. But interestingly, those infected with the Turnip Crinkle virus developed more severe symptoms than the wild type. Thus, AGO2 is known to be positively important for antiviral activity (Jaubert et al. 2011; Wang et al, 2011). However, the defense mechanism happened in multilayers where AGO1 works as a first suppressor and then is followed by AGO2 activation when AGO1 is repressed to limit virus accumulation (Harvey et al. 2011). It is because AGO2 expression is limited by AGO1 with a feedback loop effect.

All in all, AGOs are key effectors on the miRNA pathway responsible for extensive gene expression regulation, and their loss can generate a wide series of pleiotropic effects on plant phenotype. As distinct miRNAs target a various number of transcriptional factors, phenotypical changes are pronounced mostly through these. Plant development relies on a coordinated action of several transcription factors which are often regulated by the miRNA pathway implying the AGO proteins. It has been reported that the members of the plant-specific class III HD-ZIP protein family are responsible for the adaxial identity, and the triple mutant from this protein family showed needle-like leaves without blades (Emery et al. 2003; Prigge et al 2005). PHAVOLUTA (PHV) and PHABULOSA (PHB) parts of the class III HD-ZIP protein family are regulated by miR165/166 microRNAs through specific degradation of their mRNA by AGO1 (and some other type of AGOs). Disruption of this pathway by the introduction of a mismatch at the miRNA region or removal of AGO1 resulted in similar developmental defects (Mallory et al. 2004). The gradient intensity of MIR165/166 concentration at abaxial leaves also supports this suggestion where the complementary sequence between this miRNA and PHB mRNA, as well as PHV mRNA as its target influence those proteins' intensity and affects the developmental defect (Kidner and Martienssen, 2004). A similar defect on *ago1-25* and *ago1-27* single mutants has been reported with dark green rosettes and serrated leaves (Morel et al. 2002).

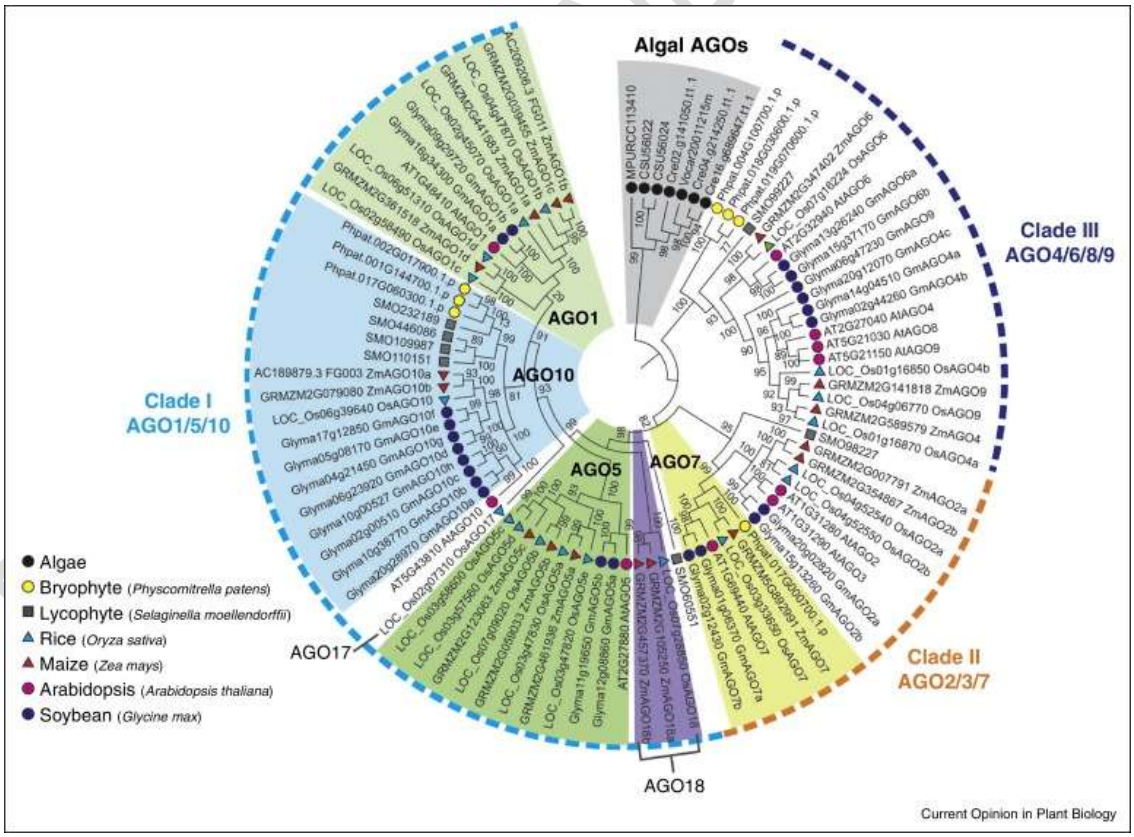

Figure 4. Phylogenetic tree of AGO proteins in plants (Han et al. 2015).

3.4 Expression pattern and sub-cellular localization of AGOs

In some cases, some AGOs can be expressed in every organ and during every developmental stage while others can be strictly expressed on a specific cell type or environmental condition. Those AGOs that express broadly in any stage of development (even at the embryo stage) or any tissues (seed, flower, apex, leaf, stem, and root) are the highly crucial AGOs to be present. AGO1 and AGO4 proteins are usually constantly inherent until the end of the plant life cycle, although their expression level varies and is highly spatiotemporally regulated. However, the other AGOs like AGO5, 6, 7, and 10 are only highly expressed during the embryo stage and followed by a limited expression in vascular strands and cotyledons, strictly on the adaxial side during the later developmental stage. In terms of AGO10, the transcription level is intense in shoot apical meristem (SAM) and adaxial primordia, repress when the leaf is young, but then it is increasing again when the leaf is getting old (Mallory et al. 2010; Jullien et al. 2022; Zhang et al. 2020).

Interestingly, AGO2 and AGO3 having considerable differences only in their promoter sequences show a common expression profile to biotic or abiotic stress conditions, which might refer to their evolutionary connection due to possible duplication events. Originally, even though the expression level is so low in a normal situation, these two AGOs are pretty much expressed mostly in siliques and seeds (Alazem et al. 2019; Jullien et al. 2020).

Indeed, the clade system that is proposed based on phylogenetic analysis shows some overlapping features with biological function, as some studies have shown a correlation between the clade and localization of the protein in the cell. Most of the AGOs under the clade I are localized in cytoplasmic, including AGO1 and 10. A recent study proposed a possible nucleo-cytosolic shuttling model of AGO1 as previously mentioned (**Fig. 3. Right**). However, the experiment also reveals a rich AGO1 localization in cytosol mostly during the G1 and S phases of the cell while an intense nuclear localization appeared only in the G2 phase. AGO10 is also mostly localized in the cytoplasm, but there is no research record for possible nuclear-cytoplasmic shuttling of it, which needs to be investigated further. Additionally, clade II, including AGO2 has dual subcellular localization while clade III has nuclear localization most of the time since the member of this clade had a major role in TGS (Law et al. 2010; Trolet et al. 2019; Iki et al. 2018).

3.5 RISC formation and AGO-miRNA binding preference

3.5.1 RISC formation

Only one strand of the RNA duplex is loaded into the AGO protein while the other strand is degraded. Strand selection is based on the thermodynamic stability of the sRNA ends. The assembly of the complex begins when the heat shock protein 90 (HSP90) dimer protein binds to AGO1 and then this triggers the binding of ATP to this complex (**Fig. 5**). This energy-containing molecule changes the conformation of AGO1 and opens the gate for miRNA duplex to be integrated. The dissociation between AGO1-containing miRNA and HSP90 occurs due to the presence of ATP hydrolysis and this dissociation triggers another change in

AGO1 conformation and leads to the removal of the unused strand, creating a mature activated RISC (Taichiro et al. 2010).

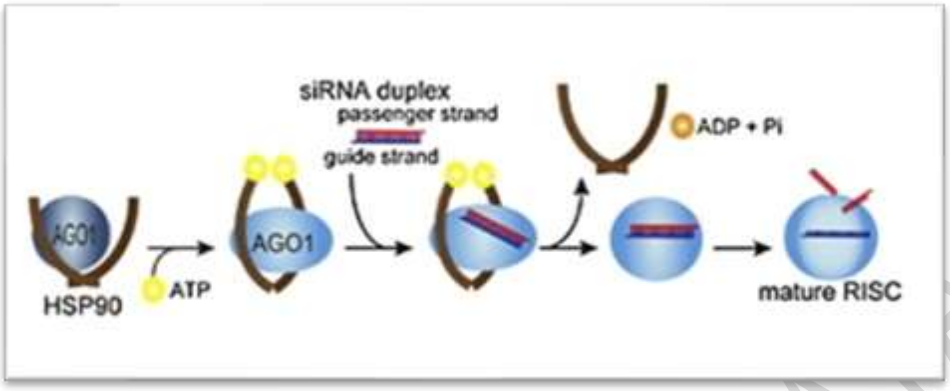

Figure 5. RISC formation (Taichiro et al. 2010).

3.5.2 Binding Preferences of AGOs

Besides the fact, that individual sRNAs can be loaded to more AGOs, different AGOs have preferences for distinct types of sRNAs. Factors to determine the faith of a given sRNA can be the 5' end nucleotide identity and the length of the mature strand. Additionally, miRNAs tend to be loaded into AGO1-containing RISCs.

The identity of the 5' nucleotide of small RNA as well as its length affects the sorting efficiency of these small RNAs into AGO proteins. A study found that AGO1 was associated mostly with small RNAs with a length of about 21 or 22 nucleotides and Uridine on its 5' end while AGO2 had a strong preference for small RNA with a length of 21 nucleotides and Adenine on its 5' end. The MID domain is the one responsible for the strong preference of AGOs for the 5' end nucleotide sequence of the mature sRNAs (Wang et al. 2011).

Moreover, sorting can also be influenced by the secondary structure of certain sRNA. AGO2 has a preference for those sRNA duplexes which contain base pairing in the 11^{th} and 15^{th} positions while those having mismatch at the 11^{th} position of the duplex are selectively loaded into AGO1. Mismatches in the 12^{th} and 13^{th} positions of miR168 are recognized by AGO10. The difference in secondary structure outcome is due to the different miRNA biogenesis pathways which are controlled by some different DCL proteins. Interestingly, different DCL-processing proves the different lengths of mature small RNAs, thus affecting their loading efficiency into AGO protein. For example, AGO1 has a stronger preference for small RNAs processed by DCL1 (the only DCL protein that contributes to miRNA maturation) (Czech et al. 2011).

Moreover, the antagonistic roles of AGO10 and AGO1 over miR168 binding can be seen especially in the case of the possible alternative configuration structures of the mir168 duplex. MiR168 with a length of 22 nt is bound mostly by AGO10 while a canonical miR168 with 21 nt in length is favored by AGO1. As a result, this AGO10-mir168 binding preference can contribute to the homeostasis of AGO1 expression regulation because the abundance of AGO1 mRNA is also controlled by mir168 (see the next chapter for

further explanation). In contrast, an overlapping function due to the existence of binding preference can be witnessed between AGO1 and AGO2. A study has found that even though AGO1 prefers 5'U miRNA, both AGO1 and 2 can work independently and redundantly with miR408 (existing in both 5'A and 5'U forms) which have a function to regulate *Plantacyanin* production. This discovery is also in line with the association between miR165/166-AGO10 and miR390-AGO7, opposing the 5' nucleotide preference's rule and opening up to the more diverse possibility of AGO-miRNA binding preferences (Maunoury and Vaucheret, 2011; Iki et al. 2018; Andrea et al. 2023).

3.6 Maintenance of the homeostasis regarding the investigated AGOs

As previously mentioned, AGOs play a major role in silencing activity with sRNA as the guide either via mRNA degradation or translation repression by blocking ribosomes on translating targeted mRNAs. One of the most important AGOs in plant development is AGO1 being a key component of the miRNA pathway. Consequently, the malfunction or insufficient concentration in both directions (over- or under-accumulation regarding the circumstances) in the cell can have serious consequences on plant life. Therefore, keeping up with the homeostasis of this protein is essential. One major tool for this is the miR168 miRNA, which targets the Ago1 mRNA. - A disturbance in the balance of miR168 and AGO1 homeostasis could affect the activities of other miRNAs associated with AGO1 negatively or positively. For instance, a sensitive low-potassium tomato (*Solanum lycopersicum*) line shows a small amount of mir168 expression under potassium deficiency which leads to the accumulation of AGO1. The increasing AGO1 content of the cells leads to the higher silencing activity of other miRNAs such as miR858, miR384, and miR530 to down-regulate their target, including genes participating in the cytokinin pathway. Low activity of the cytokinin pathway increases the production of reactive oxygen species and destroys the integrity of plant cells (Liu et al. 2021).

Regulation of AGO1 homeostasis has multiple layers besides the action of miR168 as a negative feedback regulator of *Ago1* mRNA. Expression of *Ago1* and *MIR168* genes is controlled both independently and coordinately. A study found that during the ABA and abiotic stress treatment of *Arabidopsis*, a steady level of Ago1 mRNA and protein was recorded together with an elevation of mature miR168 and its precursor but coupled with elevated promoter activity of the *Ago1* gene. It leads to a conclusion that the increased transcriptional activity of miR168 correlated highly with an elevation of the Ago1 mRNA transcription, and this resulted in the modest accumulation of AGO1 both on mRNA and protein levels. However, the expression level of both genes (*Ago1* and *MIR168*) might be influenced by different factors and pathways but could be interwind somehow, especially during stressful conditions. It has been reported that the F-BOX WITH WD 40-2 (FBW2) protein acts as a negative regulator of AGO1 protein level in an ABA-dependent manner. While FBW2 mutation affects the plant's sensitivity toward ABA, no such phenotypical alteration was recorded which is characteristic of AGO1 deficiency. This suggests that AGO1 expression regulation is controlled by many other possible different pathways (including mir168 negative feedback). On the other hand, the MIR168 promoter possessed abscisic acid-responsive element (ABRE) motifs, a target element

of the ABF transcription Factor which is a key regulator of stress-related genes. Thus, the regulation of *Ago1* and *MIR168* gene expression could come from different pathways but could correlate not only during normal conditions but also during different environmental changes (Erley et al. 2010).

Similar to AGO1 homeostasis, the homeostasis of AGO2 protein is controlled on several levels, like expressional, post-transcriptional, and post-translational levels. Indeed, the level of *Ago2* mRNA is strongly regulated by AGO1 through miR403 targeting *Ago2* mRNA (Allen et al. 2005). There are arginine-glycine RG/GR repeats in the AGO2 N-terminal domain that could easily interact with methyltransferase5 (PRMT5). In a normal condition, AGO2 protein activity is inhibited by this enzyme which provides arginine methylation to AGO2 leading to the degradation of AGO2 protein. This enzyme could also degrade AGO2 when it is loaded with sRNAs with the help of Tudor-domain protein. During infection, the two enzymes (PRMT5 and Tudor-domain) are down-regulated which causes a higher accumulation of AGO2, thus activating all sRNA whose silencing activity is necessary for strengthening the immunity system (Hu et al. 2019).

As previously mentioned, AGO10 has not only overlapping but also antagonistic roles with AGO1 in association with mir165/166, especially in shoot apical meristem (SAM) and axillary meristems (AM) modulation and differentiation (Liu et al. 2009). Mir165/166 targets the mRNA of the HOMEDOMAIN-LEUCINE ZIPPER III (HD-ZIPIII) protein group, including REVULOTA (PHV), PHAVOLUTA (PHV), and PHABULOSA (PHB), the key proteins on abaxial leaf identity and other meristem regulators. During the shoot meristem primordium development, AGO10 expression is elevated. Surprisingly, Mir165/166 which has higher loading efficiency toward AGO10 than AGO1 limits the silencing activities of that miRNAs. It is since the relationship between AGO10 and the two miRNAs depends solely on the structure of the miRNAs and independent form AGO10 catalytic activities. In this regard, AGO10 does not degrade HD-ZIPIII compared to AGO1, efficiently. Thus, despite the silencing activity, AGO10-Mir165/166 combination increases the HD-ZIPPIII expression level. This combination is also much more preferred to become the target of SMALL RNA DEGREDING NUCLEASES (SDNs) 1 and 2 than its miRNAs association with AGO1, thus promoting the accumulation of mir156/166. Moreover, during AM development (to become lateral shoots), Brassinosteroids, auxin, and light control AGO10 expression precisely which promotes its development only at a certain time and age. In young leaves, axils BRASSINAZOLE-RESSISTANT 1 and PHYTOCHROME-INTERSCTING FACTOR 4 down-regulate *AGO10* gene transcription and prevent the development of AM while in older leaves axils, AUXIN RESPONSE FACTOR 5 increase *AGO10* gene expression (Aichinger et al. 2012; Yu et al. 2017; Zhang et al. 2020).

3.7 *Arabidopsis thaliana* as a model plant

Arabidopsis thaliana is a herbaceous annual flowering plant from the Brassicaceae family which possibly originated from Eurasia or the Western Himalayas but exists in very diverse regions around the world these days, especially the northern hemisphere with a moderate temperature climate. In this region, flowering time occurs in spring, germination in autumn, and it stays in the vegetative rosette phase during

the winter. There are more than 750 ecotypes naturally, which show different development, morphology, and physiology. But the three most used *Arabidopsis* ecotypes for genetic and molecular study are *Wassilewskija (Ws)*, *Landsberg erecta (Ler)*, and *Columbia (Col)*. In this research, ecotype Col is used as a wild-type control in comparison with mutant *Arabidopsis* plants. The life cycle of *Arabidopsis thaliana Columbia*, and the shape and size of siliques, flower, and pollen grain can be seen in **Figure 6**.

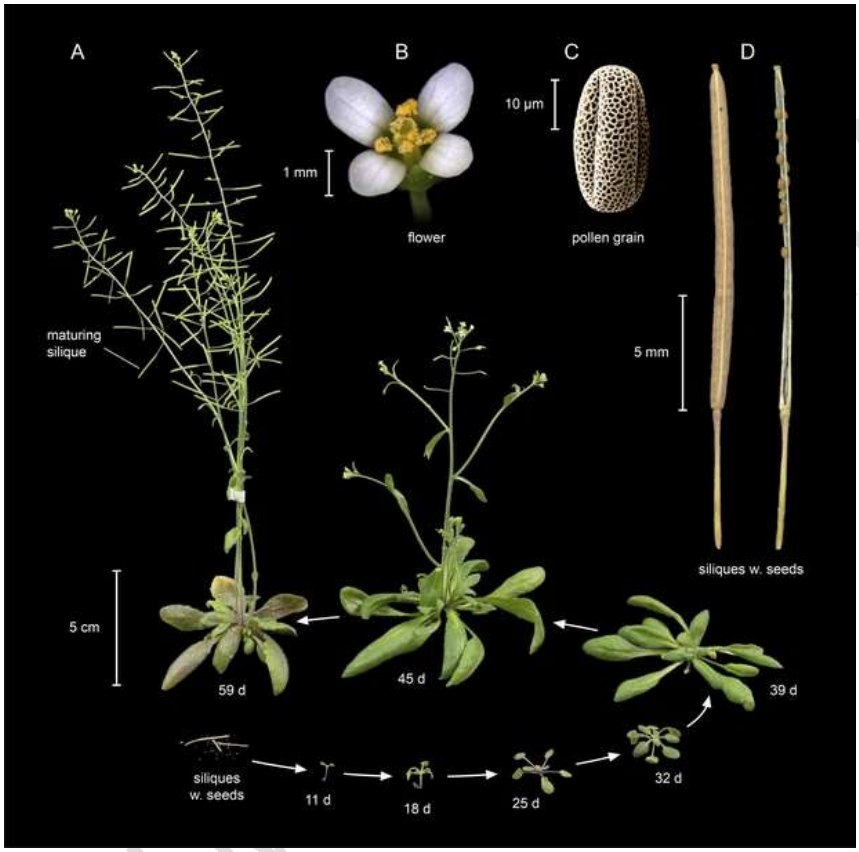

Figure 6. Life cycle, flower, pollen, and silique shape of *Arabidopsis thaliana Columbia* (Ute Krämer, 2015)

In 1943, an initiation had been proposed to use *Arabidopsis* as a model plant due to some advantageous features like fast generation cycle (5-6 weeks), small size, cross and self-fertilization possibilities, ease of growth in any controlled media, high production of seeds, a small amount of chromosome number even among other flowering plants, and flexible mutagenesis. Due to these advantages, a genetic map has been created to explore some functions deeply on the genomic level and connect morphogenesis to the genes by Random Amplified Polymorphic DNA (RAPD) and Restriction Fragment Length Polymorphism (RFLP) molecular markers. After the *Arabidopsis* genome sequence was published in 2000, enormous efforts were made to expand the knowledge related to molecular mechanisms behind plant development, genetics, physiology, epigenetics, and cell biology and to open the gate for further applications to enhance knowledge adaptation for crop plant improvement. For instance, research had been conducted in 2005 regarding the application of one particular *Arabidopsis* gene to improve the Canola plant. In the wild, *Arabidopsis* spreads its seed by a pod-shattering natural technique and these traits are controlled by the *FRUITFULL* gene. However, although this trait also occurs in Canola, the pod-shattering mechanism

is one of the most unfavorable traits from the perspective of Canola farmers. Using this knowledge, a pod shatter-resistant Canola plant was produced with ectopic expression of this gene. Another example can be seen in the attempt to understand of flowering-time of sunflowers and pearl millet (Ostergaard et al. 2006; Blackman et al. 2011; Clotault et al. 2012).

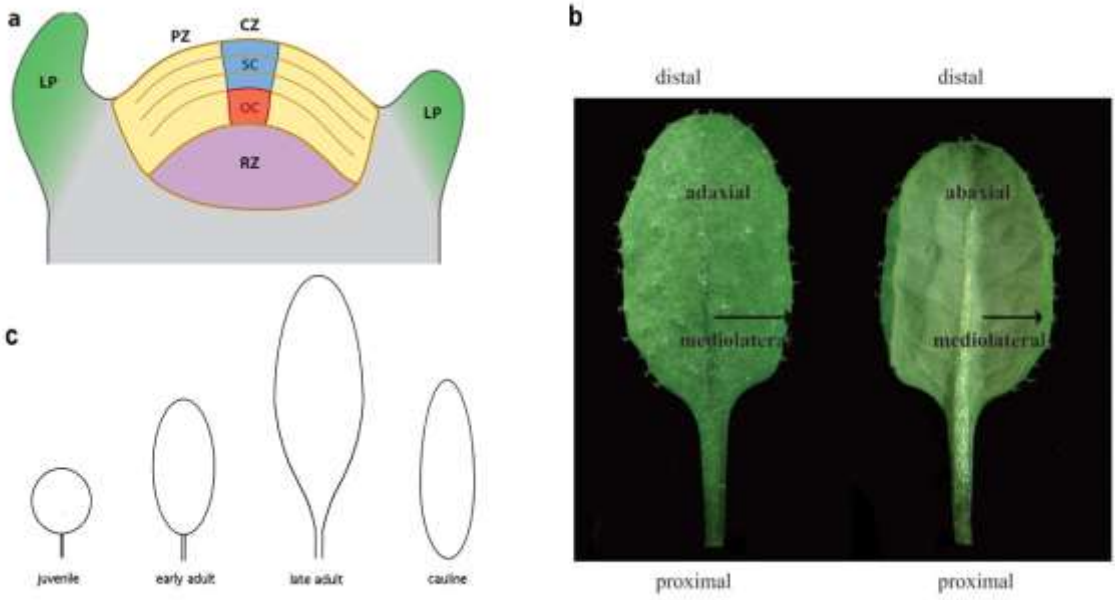

Figure 7. a. Shoot Apical Meristem, b. polarities, c. leaf shape during developmental phases of *Arabidopsis thaliana Columbia* (Aichinger et al. 2012; Redei, 1970).

3.7.1 Morphogenesis of *Arabidopsis thaliana*

After embryogenesis, the plant develops two different meristems, one to form the shoots and another to promote roots. The leaves develop from the tip of the shoot namely Shoot Apical Meristem (SAM). In this meristem, there is a central zone (CZ) with stem cells (SC), undifferentiated cells that keep division continuously at a slow speed, a peripheral zone (PZ) surrounds it with faster cell division which initiates lateral organs including flowers and leaves, and rib zone (RZ) that starts to differentiate the continuously divided cells to become central stem tissue with its flattened shape characteristic **(Fig. 7.a)**. Below stem cell layers, there is an area called the organizing center (OP) which has the function to keep the stem cells above in the form of undifferentiated cells by expressing *WUSCHEL (WUS)* which could downregulate some proteins responsible for cell differentiation (Aichinger et al. 2012).

During leaf initiation, a flanked area of the late peripheral zone occurs and this is followed by the formation of leaf primordia with three polarities including medial-lateral, proximal-distal, and adaxial-abaxial (**Figure 7. b**). During leaf primordia formation, the adaxial is located side by side with the SAM area while the abaxial is on the opposite side. However, during leaf development, the cells that differentiate in the adaxial area divide more rapidly compared to the abaxial side and thus, become the upper part of the leaves. Moreover, rounded small leaves are produced in the early phase of leaf development with distinctive petioles

and elongate to the distal polarity, becoming more oval spatula-like leaf shapes with stellate trichomes populating the adaxial surfaces in these adult leaves. On the other hand, another type of leaf forms between the main stem and inflorescence stem namely the cauline leaf shoving a small lanceolate shape and lack of petiole (**Fig. 7.c**) (Redei, 1970; Van et al. 1992).

Furthermore, a typical flower structure is existed in *Arabidopsis*, including four petals, four sepals, two carpels, and six stamens. This flower produces fruits called silique with two valves and a false septum in between. The elongation of siliques happens until 2-3 weeks after pollination (David et al. 1998; Filippo et al. 2012)

3.8 Production of *ago1*, *ago2* and *ago10* mutants and their genetic background

Some *ago1* null mutants had been successfully isolated and studied previously with intense defects in plant development including filamentous leaf structure, abnormal inflorescence, and even infertility. As null mutants are severely distorted and sterile, to study the functions of AGO1, hypomorphic mutants were produced. Two such mutants were *ago1-27* and *ago1-25.* They also show similar but not so pronounced defects such as dark and serrated leaves, late flowering, stunted appearance, and reduced flowering but still maintained fertility. In this study, we *use ago1-25, ago1-27, ago2-1,* and *ago10-1* mutants. *Ago1-25* and *1-27* are hypomorphic mutants, a term that defines an alteration of a gene product by only reducing the level of the mutated gene's activity, but not eliminating it, entirely. Full inactivation is undesirable for genetic studies, especially on the most essential genes, since it brings an instant lethality and makes it difficult to study the function of backup genes.

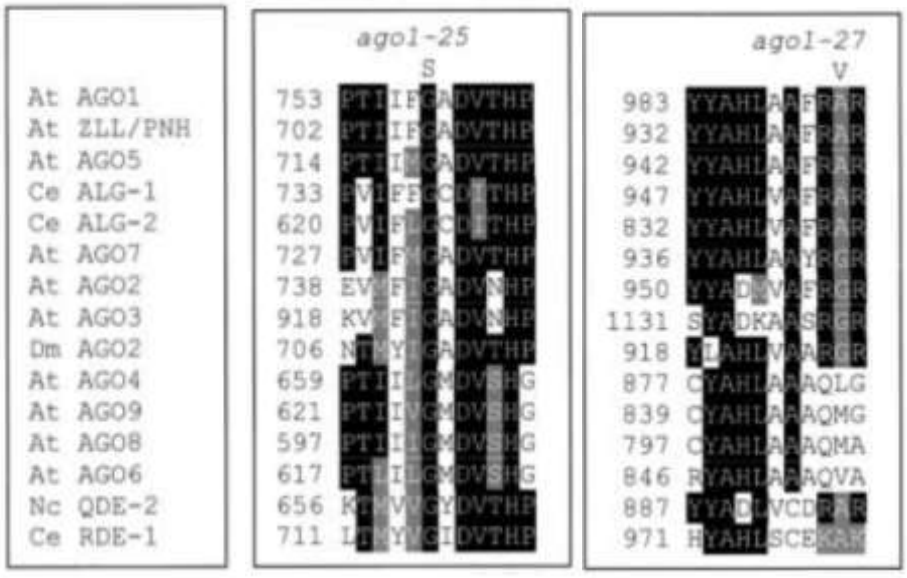

Figure 8. Single amino acid substitutions of *ago1-25* and *ago1-27* (Filippo et al. 2012).

In the *ago1-25* mutant the Gly759Ser, in *ago1-27* the Ala993Val amino acid substitution impaired the function (**Fig. 8**.). Both of the positions of the mutated amino acids (759 and 993) are located in the PIWI domain. PIWI domain is constructed by the sequences of amino acids from the position of 676 to 997 of the

AGO1 amino acids sequence which have slicing activities. Thus, this mutation is suggested to have reducible slicing activity of the AGO1 protein.

In the *ago2-1* mutant of *Arabidopsis thaliana* the protein function is destroyed by a T-DNA insertion in one of the exons. There is no phenotypic alteration concerning this mutant as previously mentioned, but the mutant is highly susceptible to tobacco rattle virus and potato virus X. Furthermore, a similar type of T-DNA insertion occurs in the *ago10-1* mutant The T-DNA insertion lines were created by vacuum infiltration of *Columbia (Col)* plants with pROK2 vector in *Agrobacterium tumefaciens* and kanamycin was used to select plants carrying the T-DNA. Each T1 transformant has been maintained individually at SALK Institute and the DNA sequence of each T-DNA flanking region was generated from seedlings grown from the same sample of seeds as that provided for distribution (T3) (José et al. 2003; Morell et al. 2002). The single mutants were provided by the research group, while the crossing was conducted to generate double and triple mutants (Zhang, 2006). In the end, 11 different mutant combinations were used in this research, including single (*ago1-25, ago1-27, ago2-1* and *ago10-1*), double (*ago1-25, 10-1; ago1-25, 2-1; ago1-27, 10-1; ago1-27, 2-1; ago2-1, 10-1*) and triple mutants (*ago1-25, 2-1, 10-1* and *ago1-27, 2-1, 10-1*). As a wild-type control, *Arabidopsis thaliana* Columbia was used.

3.9 Candidates for functional redundancy with AGO1

Current work focuses on finding substitute AGOs for AGO1 function in the miRNA pathway. AGO2 and AGO10 were selected as the subject of this study based on previously published data and experiments conducted in our laboratory. AGO2 was selected due to its overlapping role with AGO1 in *plantacyanin* production as previously mentioned, and based on our observation in terms of AGO1 deficient plants which over-express Ago2 (data not shown). As previously mentioned, AGO1 and AGO10 are together under the same clade with closely similar amino acid sequences, and there is evidence of their cooperative acting. Due to that reason, AGO10 was chosen to be analyzed in this study. Previous work of the research group (Dalmadi et al. unpublished) confirmed the anti-parallel expression pattern of Ago1 and Ago10, as plants having repressed AGO1 content due to the over-expression of miR168 showed up-regulation of Ago10 (**Fig 9**).

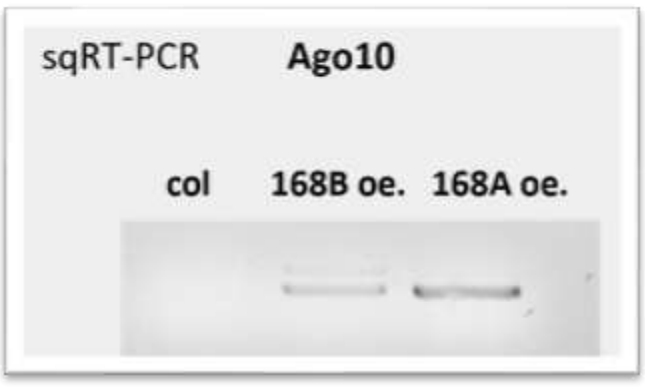

Figure 9. Semi-quantitative RT-PCR of Ago10 in mir168 over-expression plants.

3.10 Fast Protein Liquid Chromatography (FPLC) as a useful tool in the detection of RISC

This technology is based on low-pressure chromatography to nucleo-protein complexes. There are two phases in this method, as a stationary phase a column of Sephadex is used to provide the matrix, which retains biomolecules in a size-dependent manner, the mobile phase is an aqueous buffer that carries the molecules of the plant extract and protects them from degradation (Kristen and Arun, 2021). Working with fresh crude extracts at low temperatures allows it to keep nucleo-protein complexes intact (Dalmadi et al. 2019).

Determining the integrations of some specific small RNAs into protein complexes including the AGO-RISC complex can be done by gel-filtration size separation method based on FPLC. In the previous study, three pools of small RNAs were identified in *Arabidopsis taliana*, where AGO1-RISC (high molecular weight RISC) contained miRNAs, AGO4-RISC (low molecular weight RISC) associated with 24-nt-long siRNAs and the third pool composed of protein-unbound sRNAs. Moreover, some miRNAs have different loading efficiencies in the RISC complex. It has been found that miR168, miR157, and miR390 have a very inefficient loading capability into RISC in leaves, while miR319 and miR159 possessed high efficiency. It has been shown also that miR161 and miR167 show intermediate loading efficiency (Damadi et al. 2019). Thus, with this technology, we could visualize the loaded and unloaded miRNAs parallel from the same tissue.

4. MATERIALS AND METHODS

4.1 Plant Material and Growth Conditions

Single mutants were provided by the research group and originated from the NASC seed bank. Double and triple *ago* mutants were produced by the crossing method of *Arabidopsis thaliana.* F1 progenies of the crossing were tested for their heterozygous nature and self-pollinated. Homozygous plants were selected and left to set seeds. Current work used the next generations. The seeds were surface sterilized with hypochlorite and germinated *in vitro* on MS medium (Duchefa premix) for one week under continuous light and a constant 21 °C. Seedlings were then kept in peat blocks for further 3 weeks under short day (8 hours light) conditions at 21 °C, planted into pots, and placed on long days (16 hours a day). The phenotypic observation was conducted at the indicated time points (3, 5, 11, and 16 weeks old).

4.2 Direct PCR

The existence of mutations was detected with direct PCR using crude extract of plants. An approximately 1 mm² leaf sample was homogenized in 20 ul of extraction solution with a pipette tip. Samples were incubated at 95 °C for 15 minutes, and placed on ice. 20 ul of dilution solution was then added, and after a brief vortex, they were centrifuged for 1 minute. A supernatant was used to make the PCR.

Reaction composition was the following: 1 ul crude extract as a template, 1 ul of each of the three primers, 0.1 ul Phire Taq polymerase, 0.2 ul of 10 mM dNTP solution, 2 ul Phusion buffer mix, and 3.7 ul MQ water. Individual reactions were set up simultaneously from a PCR mix. To detect *ago2-1* mutation, Ago2-1_R, Ago2-1_F, and LBb1_SALK primers were used (see Supplementary materials), the latest targets transposon T-DNA insertion of AGO2 locus. To detect *ago10-1* mutants, Ago10-1_R, Ago10-1_F, and LBb1_SALK primers were used. PCR conditions were the following: 98 °C for 2 minutes as initial denaturation, 34 cycles of 98 °C for 10-second denaturation, 64 °C 20 seconds of annealing, and 72 °C 30 seconds extension, as well as 72 °C for 5 minutes as a final extension. The separation and detection of amplified PCR results can be done by using gel electrophoresis with 2% agarose gel and Ethidium bromide. Images were taken with ChemiDoc equipment using the Image Lab program.

4.3 Total RNA extraction

Extraction buffer was made by mixing 2 ml 10% SDS and 10x Extraction buffer (0.1 M glycine–NaOH, pH 9.0, 100 mM NaCl, 10 Mm EDTA, 2%sodium dodecyl sulfate, and 1% sodium lauroylsarcosine) into 7 ml MQ water for 10 ml extraction buffer. The whole rosette of 8 weeks plant was used to prepare a common phenol-chloroform extraction. Plant materials were homogenized in a cold mortar in 650 ul/ 200 mg sample extraction buffer. 650 ul of the extract was used to purify nucleic acids. Samples were purified in subsequent steps using 600 ul of phenol, phenol-chloroform 1:1 solution, and chloroform. The final supernatant was pipetted into Eppendorf tubes containing 20 ul 4M Na-acetate and 1 ml 96% ethanol and precipitated in the

deep freezer (-70 ^{0}C) for a minimum of 1 hour or overnight. Samples were centrifuged for 30 minutes at 4 °C, the nucleic acid pellet was washed twice with 70 % ethanol, dried in Speedvac equipment, and dissolved with 30 ul sterile water. The total nucleic was stored in the freezer. The quantity and quality of the total nucleic acid were measured using agarose gel and Nanodrop.

4.4 Northern blotting for small RNA detection

6 µg of RNA was loaded onto urea containing acryl-amide gel. Acrylamide gel is made with the composition of 20 gr Urea, 4 ml (RNA) Acrylamide solution, 16 ul TEMED, 240 ul APS.and MQ water which is added to get the total volume of 40 ml. Separation is conducted in a vertical electrophoresis tank at 450 volts. RNA was blotted onto the Hybond NX membrane (GE Healthcare) with a semi-dry blotter. Chemical cross-link was used to immobilize RNA on the membrane using a mixture containing 10 MQ water, 122,5 ul methyl imidazole, 10 ul hydrogen-chloride, and 373 mg of 1-Ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride. The wet membrane should be covered with foil and incubated for 1 hour at 60 °C. Hybridization was carried out with biotinylated LNA (miR159) or DNA (miR168) probes at 50 or 37 °C, respectively. Detection was based on Thermo Scientific North2South chemiluminescent Hybridization and Detection Kit instruction and the blotted hybridized membrane were detected by the ChemiDoc XRS imaging system.

4.5 Semi-quantitative RT-PCR

2 µg of total nucleic acid samples were treated with DNase I to eliminate genomic DNA according to the instruction of the manufacturer (NEB), then further phenol-chloroform purification was carried out. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fischer Scientific) as described.

For the PCR 1 ul of cDNA, 1 ul of the desired primers, 0,2 ul Phire enzyme, 0,4 ul dNTP, and 4 ul buffer was added, and 12,4 ul water or up to the total volume of 20 ul. UBC9 is used as a reference gene with PCR setting as followed: 98^{0}C (30 sec) for initial denaturation, 98^{0}C (10 sec) for denaturation, 64,7^{0}C (10 sec) for annealing, and 72^{0}C for (20 sec) extensions with all the three stages set to perform 25 cycles, and 72^{0}C (2 min) final extension. mRNA that was desired to be analyzed was Argonaute 1 with primer AGO1_500 Forward and Ago1_814 Reverse (Supplementary material) with PCR setting as followed: 98^{0}C (2 min) for initial denaturation, 98^{0}C (20 sec) for denaturation, 60^{0}C (20 sec) for annealing, and 72^{0}C for (25 sec) extensions with all the three stages set to perform 25 cycles, and 72^{0}C (5 min) for final extension. The cDNA amplification reaction was separated with agarose gel in the presence of EtBr. The gel was detected with the ChemiDoc XRS imaging system. The detected signal was measured using ImageLab 5.2.3 software of Chemi-Doc equipment from BioRad and Ago1 cDNA amplification result was normalized with Ubc9 result with the same software.

4.6 Gel-filtration with FPLC

Rosette leaves of 5 weeks old plants (0,3 g) was homogenized in 600 µl of ice-cold FPLC buffer (50 mM Tris–HCl pH 7.5, 10 mM NaCl, 5 mM MgCl2 and 4 mM DTT)), and centrifuged at 4 °C for 4X10 minutes at 15 000 RPM to avoid cell debris. The supernatant was moved every time into a new sterile tube. 200 µl of the cleared extract was loaded onto the column, and was separated in FPLC buffer with 0,15 ml/minute flow rate at 4 °C. 48 fractions were collected and rendered into odd and even series. Both of the series were used to extract RNA with the phenol-chloroform method. The fraction containing purified total RNAs was then used for the northern blot with the method previously described.

5. RESULTS

5.1 Phenotypic alterations of the investigated mutants

5.1.1 Differences Observed in the Development

As for the cotyledon, normal development can be observed in the case of *ago2-1, ago10-1,* and *ago2-1, 10-1* mutants (**Fig. 10)**. In this early stage of life, the presence of a mutation in any of the investigated AGO genes has not altered the leaf shape drastically. However, all plants with *ago1* mutation showed slower growth and smaller cotyledon compared to the wild type and appeared to be slightly darker green. Three weeks after sowing, *A. thaliana Columbia* wildtype, *ago10-1* single mutant, and *ago2-1, 10-1* double mutant have shown to possess four young leaves.

Eleven weeks old plants show visible variations in their general appearance (**Fig 11**). All of the plants having *ago1* mutation have difficulties in paraclade formation, as they developed less rosette or cauline-initiated and accessory paraclades. A lack of plant enlargement most severely appeared in *ago1-25, 2-1, 10-1* triple mutant and *ago1-25, 2-1* double mutant. In contrast to this, both *ago2-1, ago10-1* and their double mutant is rich in paraclade growth, cauline leaves and even inflorescence stems and flowers. Surprisingly, even though all *Columbia* wildtype*, ago2-1, ago10-1,* and their double mutant almost reached the end-of-life cycle in the next 16 weeks, dried and wilted, the mutant lines having *ago1* were still alive and slowly kept flowering, especially *ago1-27, 2-1, 10-1* and *ago1-25, 2-1, 10-1* triple mutants. At this stage, all *ago1-25* and *ago1-27* single and double mutants possessed several paraclades initiated from the middle of the rosette and the cauline. On the other hand, *ago1-25, 10-1* double mutant developed a single main stem without paraclades.

Moreover, the speed of development was completely reduced in the mutants having the *ago1* mutation, and the life span of these mutants was surprisingly longer than those without the *ago1* mutation and *Arabidopsis thaliana Columbia* wildtype.

5.1.2 Alterations of the rosette and cauline leaves

As for the fully developed rosettes of mature plants, only faint alterations could be detected taking the *ago10-1* and *ago2-1* single mutants compared to wildtype *Columbia* **(Fig. 12a).** A lighter green color of leaves was characteristic to both of the mutants compared to *Columbia* wildtype, while a slight over-growth of abaxial surface of *ago10-1* mutant created a slight downward-curled shape of leaves toward lateral expansion. Interestingly, *ago1-25* and *ago1-27* single mutants clearly showed some defects in the formation of leaf blade shapes and polarity on the rosette and produced narrow radial rosette leaves. Specifically, a curly-U shape feature can be seen on the rosette leaves, representing adaxial surface overgrowth and rather moderate growth on lateral expansion. An extreme case of similar features mentioned can be found specifically on *ago1-25*, describing a longer growth toward the distal direction. Moreover, these two single

mutations manage to alter the defined petioles which should be seen in the wild type of *Arabidopsis thaliana* and produce a more distinctive spiky blade of the leaf.

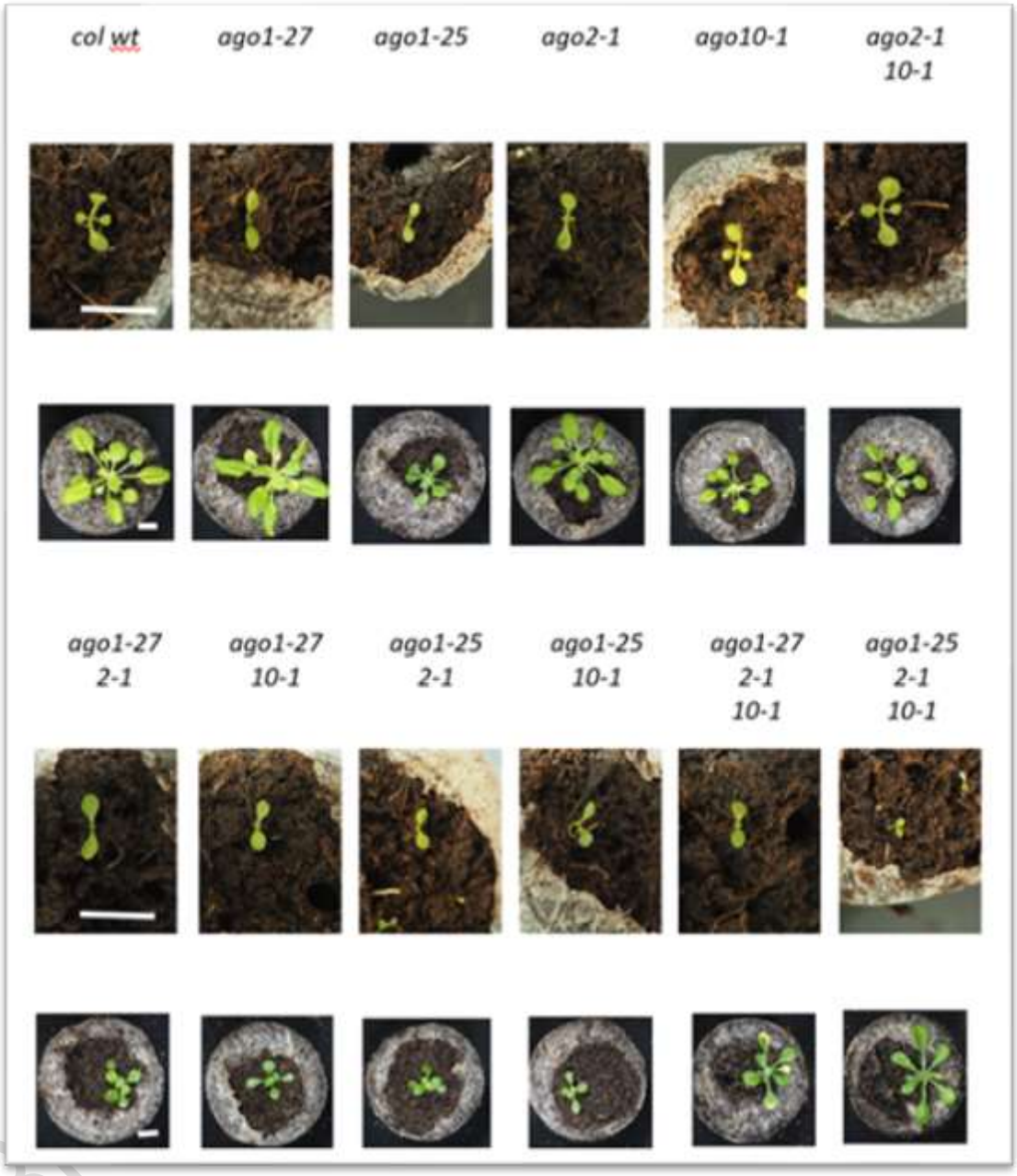

Figure 10. Early phenotypes of the investigated *ago* mutants

Phenotypic appearance of three weeks old (first line of each mutant) and five weeks old (the second line of each mutant) plants. The white line represents 1 cm.

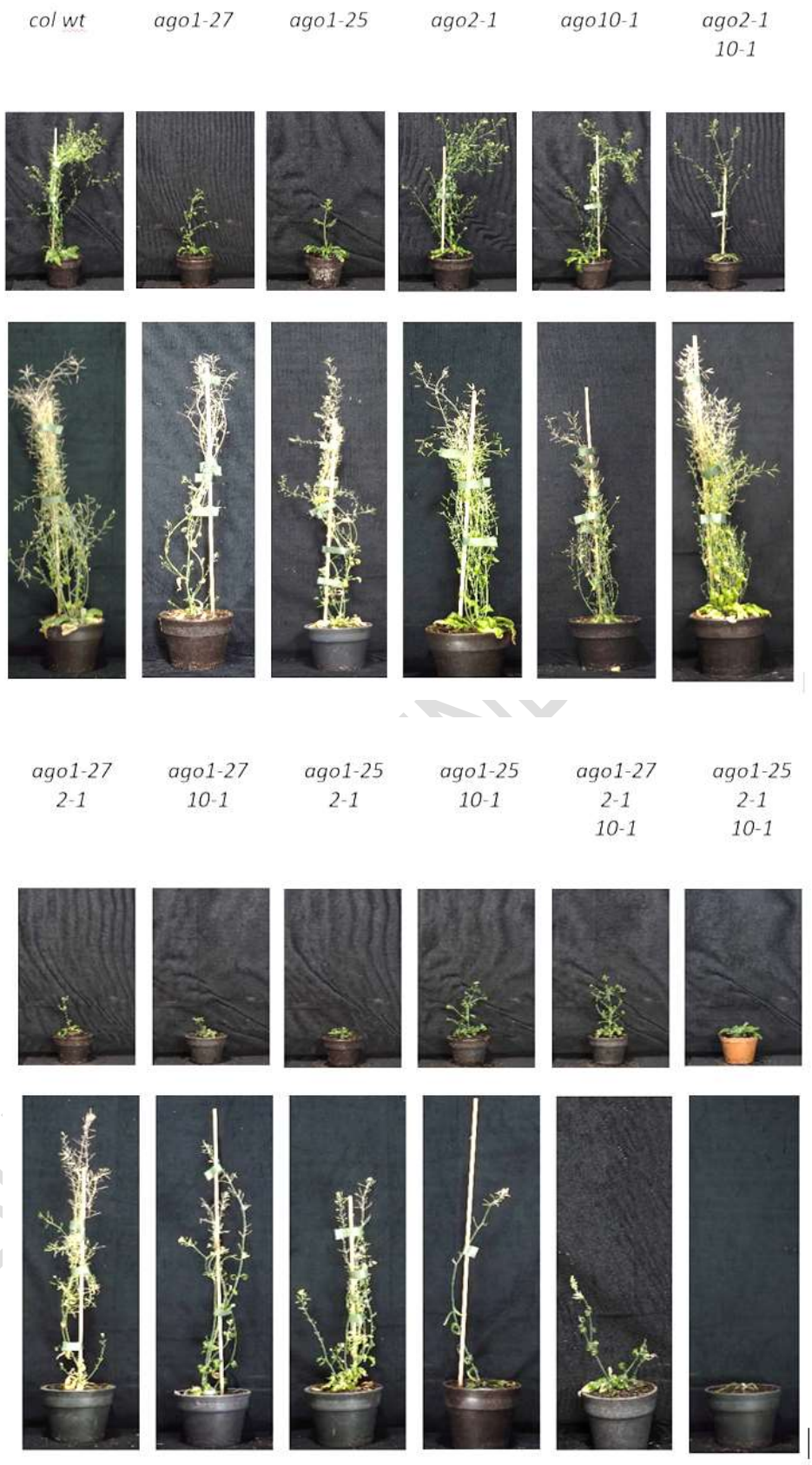

Figure 11. Late phenotypes of the investigated *ago* mutants

The general appearance of eleven weeks old (the first line of each mutant) and sixteen weeks old (the second line of each mutant) plants.

Lines having an *ago1* mutation can be easily distinguished from the other based on typical alterations of the rosette in the late phenotypes of the investigated mutants. A pungent-like edge and valley-shaped leaves can also be witnessed altering hydathodes into a sharper shape. Moreover, an extreme outgrowth of the adaxial side on the rosette leaf can be seen particularly on *ago1-27, 2-1* double mutant while structures similar to succulents are, in fact, dominant across almost all double mutants with *ago1* mutation **(Fig. 12a).** In these cases, rosette axis grows asymmetrically showing disruption of normal rosette formation as seen typically on the mutant having *ago1* (*ago1-27* single mutant on **Fig. 12a**). Interestingly, darker green color not only appeared on plants containing the *ago1* mutation but also on *ago2-1, 10-1* double mutant. This later showed the absence of lateral expansion, constructing very long and thin radial-shaped rosette leaves. Surprisingly, both triple mutants manifest more severe alterations with different features. *Ago1-25, 2-1, 10-1* triple mutant possesses longer distal expansion of leaves with needle-like rosette structure similar to *ago1-25* single mutant and even sharper spiky blade of leaf compared to another triple mutant line. The two triple mutants still maintain a common feature possessed by other mutants containing the *ago1* mutation, however, with darker green, U-shaped leaves and asymmetrical growth of rosette (for U-shaped leaf, see the arrow on **Figure 12a**).

In terms of *ago1-25, 2-1, 10-1* triple mutant multiple cauline-like leaves grow and gather in a single spot, showing incapability of the inflorescence meristem to localize phytomer or arrange node growth sequences **(Fig. 12b** *ago1-25, 2-1, 10-1* triple mutant**).** On the other hand, the cauline leaves of *ago1-27, 10-1* double and *ago1-27, 2-1, 10-1* triple mutants express an extreme roll-up leaf extension while *ago1-25, 2-1* double and *ago1-25, 2-1, 10-1* triple mutants still maintained rough spiky blade leaf similar to the corresponding rosettes.

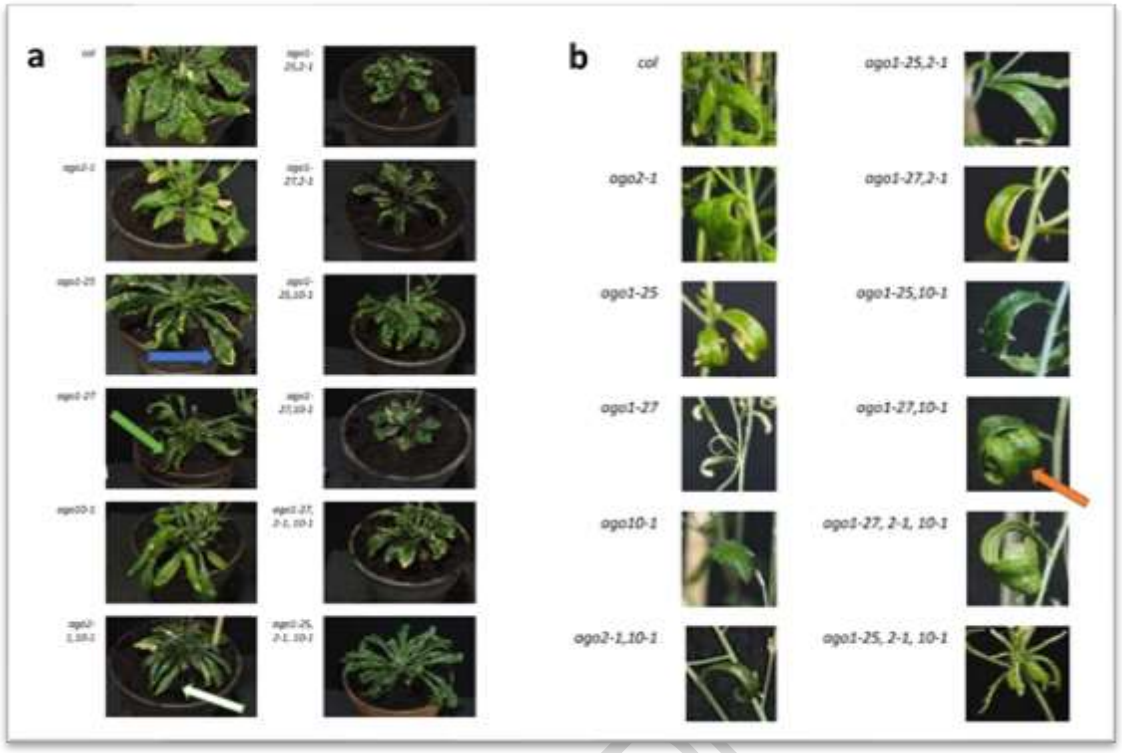

Figure12. Rosettes and cauline leaves of the different *ago* mutants
(a) Fully developed rosettes of the 11-week-old mutant plants. The blue arrow in *ago1-25* indicates the distinctive spiky blade leaf, the green arrow in *ago1-27* represents a U-shape curled leaf (upward curled), the white arrow showed a slightly darker green with a long and thin radial rosette in ago2-1, 10-1 double mutant (b) cauline leaves of the mutants. The red arrow in this figure represented an extremely curled-up cauline leaf.

5.1.3 Alterations of inflorescences, flowers, and silique formation

All of the mutants with the *ago1* mutation also showed low fertility, especially triple mutants. Moreover, silique shape and size were also altered in some mutants containing *ago1* where bulky crescent-like shape and reduced length are dominating alterations. The most severe silique formation defect can be seen on *ago1-25, 10-1* double mutant with dwarf silique compare to other mutants (**Fig.13a**). Furthermore, a typical flower structure existed in *Arabidopsis thaliana* Columbia wild-type, including four petals, four sepals, two carpels, and six stamens (**Fig. 13b**). The same features can also be seen on *ago2-1*, *ago10-1* single mutant, and *ago2-1, 10-1* double mutant. it also can be seen that the petal and sepals of these mutants have similar wide shapes to *A. thaliana* Columbia wild-type. Interestingly, a specific alteration in the flower organ can be seen in the mutants having the *ago1* mutation. *Ago1-25* and *ago1-27* single mutants possessed a more narrowed shape of petals and sepals. Moreover, petals in the *ago1-25* single mutant exclusively showed a longer petal while the *ago1-27* single mutant specifically happened to have longer sepal. The longer sepal however also existed on every double mutant with *ago1* mutation and triple mutant with smaller petal shape except *ago1-27, 10-1* double mutant. interestingly, *ago1-25, 2-1, 10-1* triple mutants produced a disorganized flowering pattern development. Some of the flowers were hardly distinguished, hidden under other flowers in overcrowding one single spot.

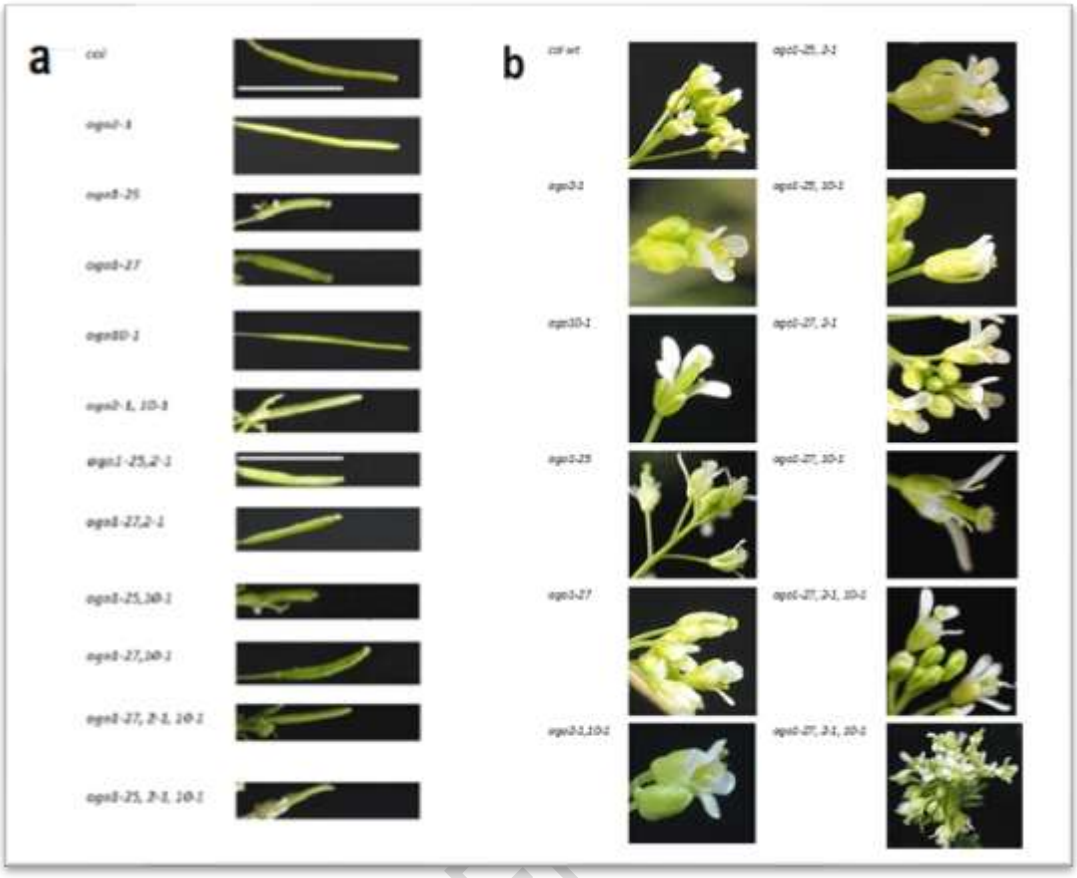

Figure 13. Silique and Flower formation of the investigated *ago* mutants. **(a)** Siliques of the mutants the white line represents 1 cm in length. **(b)** Mixed flowers and flower buds of the inflorescences of 16 weeks old plants.

Morphologically, the alteration of flower development can be seen clearly on *ago1-25* triple mutants. White arrows indicate the distorted structures. A compound cyme-like flowering pattern formed in one of the inflorescences instead of the usual raceme pattern of *Arabidopsis* where some flowers develop and overcrowd one point of the inflorescence stem without proper separation growing pattern between flowers (raceme inflorescence pattern) (**Fig. 14a, b, f, g, and i**). A near similar formation also appeared on *ago1-27, 2-1* double mutant while other lines having *ago1* mutation still slightly maintained the raceme inflorescence formation, showing a small capability on performing normal inflorescence shape (**Fig. 14c**). A fascinating stem, a term defines a flattened ribbon-like structure was also formed from the two-triple mutant inflorescence **(Fig.14 d**, **b, e**, and **h).** An abnormal U-shaped stem with backward growth that grew toward the center of gravity formed in ago1-27, 2-1, 10-1 triple mutant.

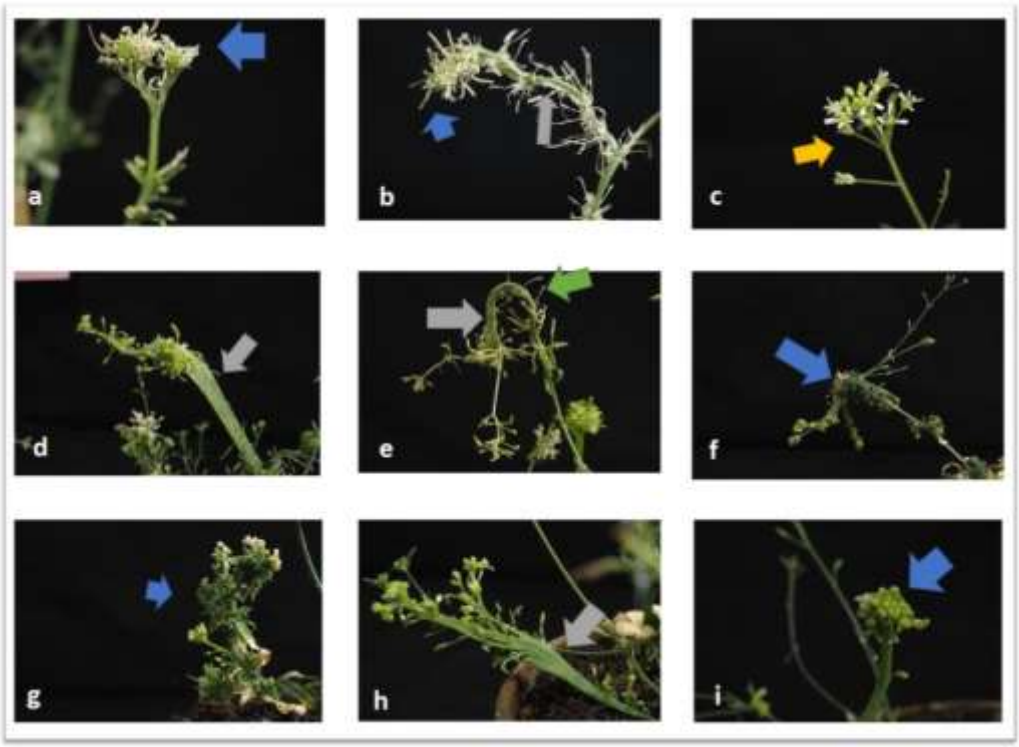

Figure 14. The late phenotype of generative organs

Generative organs of 20 weeks old **(a-c)** *ago1-27, 2-1, 10-1* and **(d-i)** *ago1-25, 2-1, 10-1* triple mutant plants. The blue arrow represents a compound cyme-like inflorescence structure, the grey arrow shows a flattened ribbon-like stem structure. The green arrow shows a U-shaped stem. The yellow arrow represents normal raceme inflorescence.

In the late phase of life triple mutants developed some extremely altered inflorescence meristems. With the gradual shortening of floral internodes, inflorescences became reminiscent of umbelliferous flowers. This phenomenon was particularly visible in the case of *ago1-27, 2-1, and 10-1* triple mutants. In a later phase, the flower meristem became completely disorganized creating a wide fascinated inflorescence stem, a tissue that consisted of an abnormal fusion of organs on a flattened ribbon-like structure with an abstract pattern of raceme along the stem **(fig. 14b, d, e, and h).** These structures had their maximum extremities in *ago1-25, 2-1, and 10-1* triple mutants. Some of the secondary inflorescence stems kept the normal pattern of raceme however, with minor defects on the tip of the stem showing a cyme-like feature **(fig. 15c).** In addition, strong defects appear on triple mutant containing *ago1-25* where all of the inflorescence stems are fascinated with abnormal umbelliferous-like structures one side of the fascinated stem **(fig. 14d and 14h).** One of the main inflorescences stems even hardly maintained its axis and created a U-shape structure with an undefined secondary inflorescence stem pattern in the tips **(fig. 14e)**. Another stem on ago*1-25* even failed to maintain its growth from the main stem and initiated abnormal stems from the central unorganized tissue **(fig. 14f and 14g).** Together these severe defects of flower stems resulted in extremely low seed production of the triple mutants. The rare siliques contained no more than one seed.

5.2 Molecular analysis of single, double, and triple mutants of Ago1, 2, and 10 genes

5.2.1 Detection of mutant alleles with direct PCR method

Before further molecular analysis, the plants were tested to prove the existence of mutations in their homozygous form. Due to the obvious phenotypical changes of *ago1-25* and *ago1-27* described previously, the single point mutations of this locus were not tested on the DNA level, and further investigations relied on the phenotype. Other loci were tested with direct PCR utilizing Phire Taq DNA polymerase resistant to reaction inhibitors inherent in crude plant extracts. Primers were listed in the Supplementary Materials section. As *ago2-1* and *ago10-1* were created with T-DNA insertion, plants were tested with three primers, two of them specific to the flanking region of the T-DNA insertion and one targeting the T-DNA itself. This way, a co-dominant genetic marker was created to give different sizes of amplicons depending on the occurrence of T-DNA insertion. PCR results correlated with the expected genotypes and showed a homozygous state in every case allowing further molecular analysis to be carried out with these plants and their later progenies**. Fig. 15** and **16** below show the result of direct PCR to detect the presence of desirable mutations.

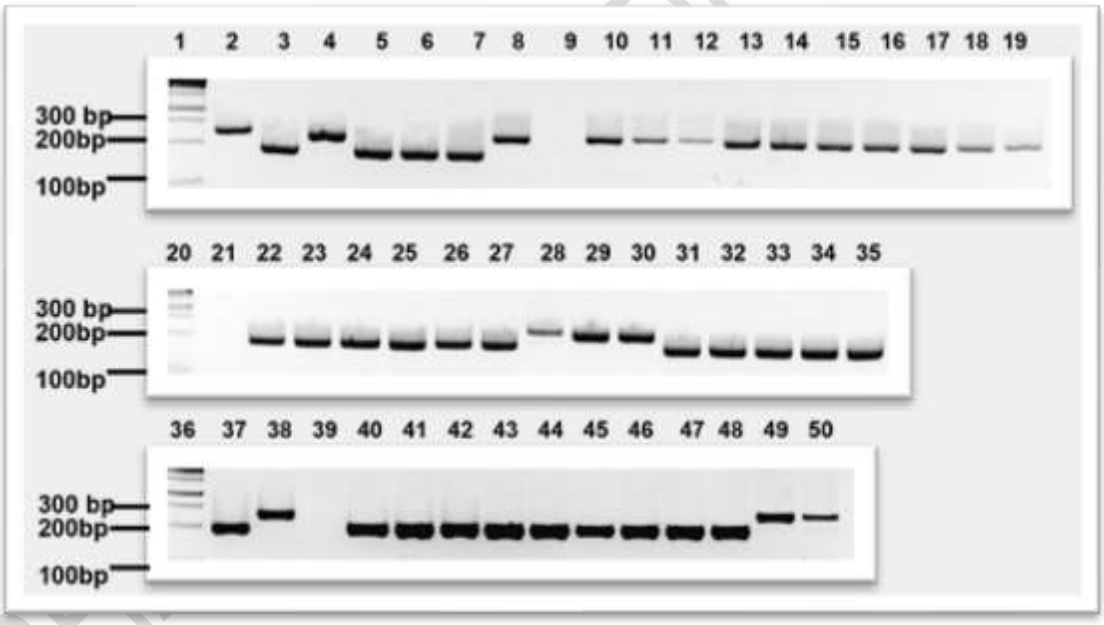

Figure 15. Direct PCR of single, double, and triple mutants to detect *ago2-1* mutation

Ago2-1 forward and reverse primers pair and LBb1_SALK primer were used. GeneRuler 100 bp DNA ladder is used and can be seen in the first section of the gel. The number shown in the figure represent as followed: **1, 20,** and **36** for the DNA ladder, **9, 21,** and **39** for negative control, **2** for *Arabidopsis Columbia* wildtype, **3** for ago*2-1* **4** for *10-1* **5-7** for *ago2-1, 10-1* **8** and **10** for ***ago****1-25* **11-13** for *ago10-1* **14-16** for *ago1-25, 10-1* and **17-19** for *ago1-27*. **22-24** for *ago1-25, 2-1*, 2**5-27** for *ago1-27, 2-1*, **28-30** for *ago1-27, 10-1*, **31-33** for *ago1-25, 2-1 ago10-1* and **34-35** for *ago2-1*. **37** for *ago2-1*, **38** for Arabidopsis Columbia wildtype, **40-42** for *ago1-25, 2-1, 10-1* **43-48** for *ago1-27, 2-1, 10-1*, **49-50** for other Arabidopsis Columbia wildtypes.

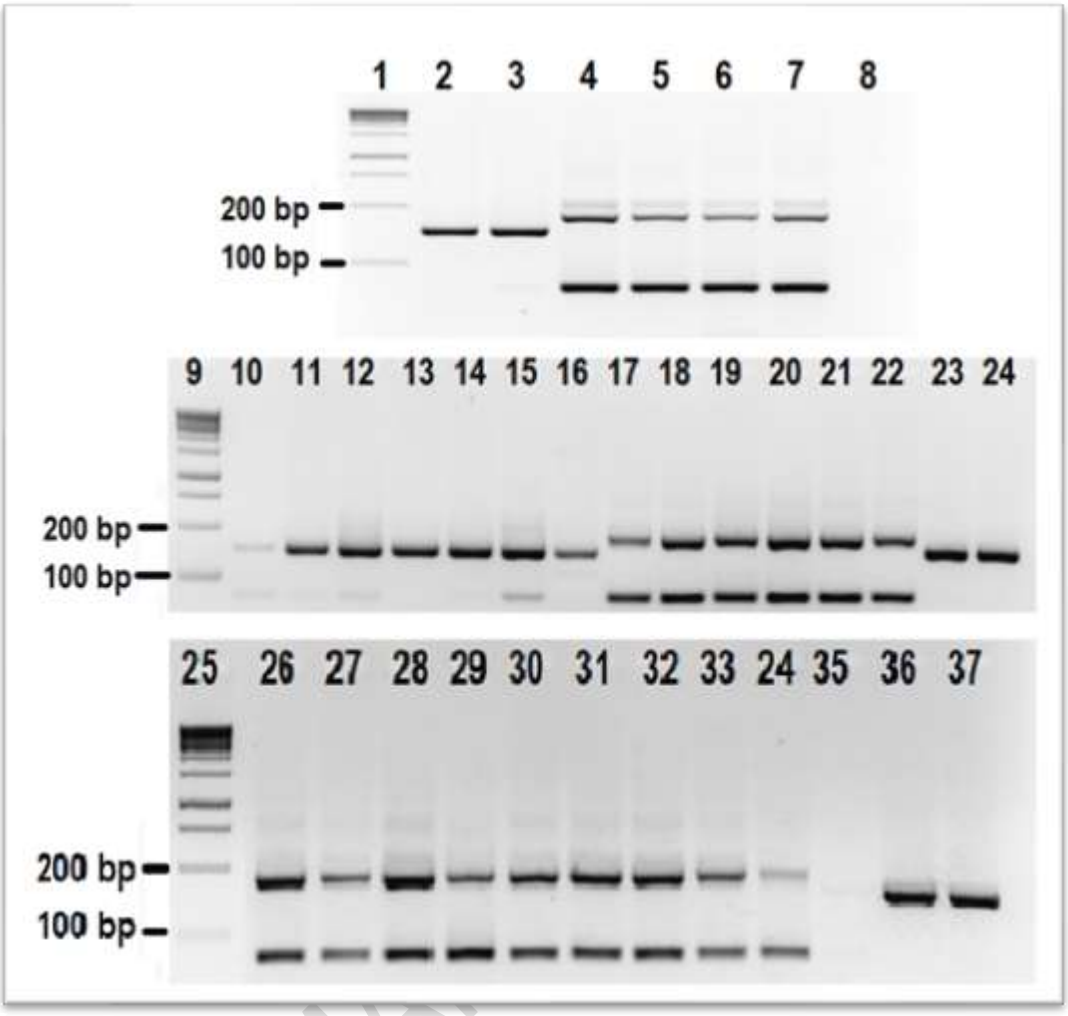

Figure 16. Direct PCR of *ago1, 2* and *10* mutants to detect *ago10-1* mutation

Ago10 forward and reverse primers pairs and LBb1_SALK primer were used. GeneRuler 100 bp DNA ladder is used and can be seen in the first or last section of the gel. The numbers shown in the picture represent **1, 9, and 20** for 100bp DNA ladder, **8, 10** and **35** for the negative control, **2 for** *Arabidopsis Columbia* wildtype, **3.** for *ago2-1*, **4.** for *ago 10-1*, **5-7** for *ago2-1, 10-1*, **11-13** for *ago1-25, 2-1* **14-16** for *ago1-27, 2-1*, **17-19** for *ago1-27, 10-1,* **20-22** *for ago1-25, 2-1, 10-1* and **23** and **24** for *ago2-1*. **26-31** for ago*1-27, 2-1, 10-1*. **32-34** for *ago1-25, 2-1, 10-1*, **36** for *Arabidopsis Columbia* wildtype, and **37** for *ago 2-1*

Ago2-1 forward and reverse primers pair and LBb1_SALK primer that was used to detect the genotype of *Ago2-1* gene produced amplicon size around 158 bp for *ago2-1* allele and 210 bp for wild type allele. *Ago10* forward and reverse primers pairs together with LBb1_SALK primer detected the T-DNA insertion of this locus with a 168 bp amplicon, while the wild type amplicon was 137 bp.

5.2.2 Detection of different miRNAs in the case of the mutant plants

As previously described, AGO1 stabilizes some miRNAs and protects them from degradation when loaded into the RISC complex. To investigate whether other AGOs can bear similar functions, above mentioned double and triple mutants were tested for their miR168 and miR159 content with small RNA northern blot. Samples were collected from the whole rosettes of 8 weeks old individuals at the same time point to ensure that all plants were in the same vegetative phase. As the mutations often caused severely

distorted phenotype leaf sample collection from the same developmental phase was rather difficult which is why whole rosettes were used. Total nucleic acid was extracted based on the mentioned procedure, concentration was measured with Nanodrop equipment, and RNA integrity was checked with agarose gel electrophoresis (**Fig. 17).**

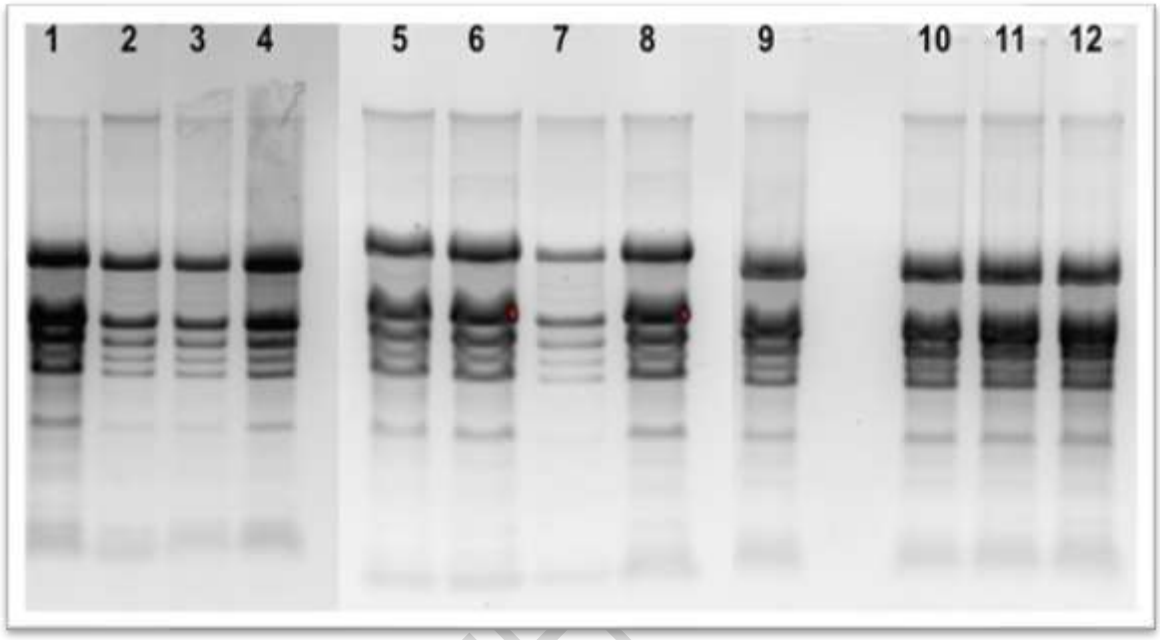

Figure17. Quality checking of the RNA extraction

The numbers indicate **(1)** *Arabidopsis thaliana Columbia* wildtype, **(2)** *ago2-1, **(3)** ago10-1*, **(4)** *ago1-27*, **(5)** *ago1-25,* **(6)** *ago1-25, 10-1*, **(7)** *ago1-27, 10-1,* **(8)** *ago1-27, 2-1,* **(9)** *ago1-25, 2-1,* **(10)** *ago2-1, 10-1,* **(11)** *ago1-25, 2-1, 10-1* and **(12)** *ago1-27, 2-1, 10-1*

5.2.3 Small RNA Northern blot

To detect miR168 and miR159 on the total RNA of mutant plants small RNA northern blot was performed. Hybridization was conducted using specifically designed biotinylated nucleic acid probes for chemiluminescent hybridization and targeting the two microRNAs. During the hybridization, a U6 detection probe was also used as a reference. The result was normalized using Image Lab software and was represented relatively to wild-type *Arabidopsis thaliana*. The following figures show the result of hybridization detection and the total miRNA accumulation for certain mutants in the form of a graph.

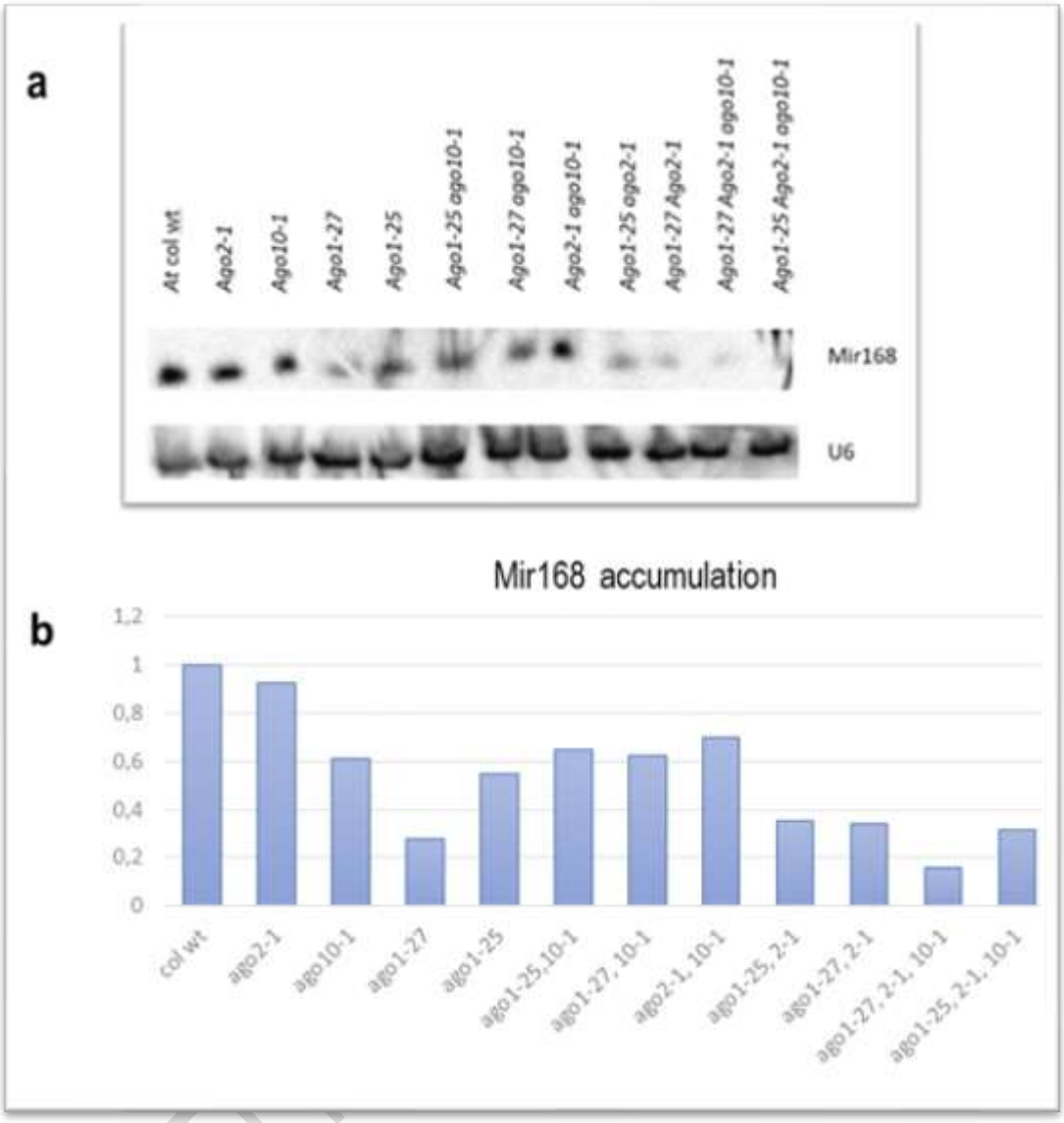

Figure 18. The accumulation of mir168

(a) Northern blot detecting miR168 amongst the investigated mutant plants. As an internal control, U6 was used. (b) MiR168 content of the mutant plants relative to *Arabidopsis thaliana Columbia* wild type. Data were normalized to the U6 level, respectively.

This experiment was conducted to reveal a possible shared role among AGO proteins in stabilizing functional miRNAs in the plant. Based on this result (**Fig 18**), mutation in any of the investigated AGO proteins caused a reduction in the miR168 level but to a different extent. In detail, a slight reduction can be seen on the *ago2-1* mutant compared to *Arabidopsis thaliana Columbia* wild type. However, the following mutants showed some noticeable reduction. In *ago10-1* the accumulation decreased to around three quarters as in the normal wild type. The double mutant having both *ago2-1* and *ago10* mutations presented a slight reduction compared to the *ago2-1* mutant, but not as low as the *ago10-1* single mutant. It seems that both AGOs act independently but could influence the accumulation of mir168 even in the presence of functional AGO1.

It can also be noted that when AGO1 is repressed, there are some noticeable disturbances in mir168 stabilization compared to the plants without the *ago1* mutation. However, to see whether there is a role of AGO2 and AGO10 in stabilizing mir168 content in the suppression of AGO1 protein, double and triple mutants having *ago1* mutation could be taken into consideration.

Compared to the single mutants *ago1-25* and *ago1-27* an intense reduction of miR168 accumulation can be detected in *ago1-27, 2-1,* and *ago1-25, 2*-1. This suggests a redundant role of AGO2 in stabilizing miR168 and proposes a substitute role for the AGO1 function in the miRNA pathway. A similar effect was detected in the case of the comparison of *ago1* single and *ago1, 10* double mutants. Both of the double mutants having *ago1-27* showed a relatively equal amount of mir168 accumulation. Interestingly, an intense decrease in miR168 content can be noticed in a triple mutant having an *ago1-27* mutation, which shows less capability of other AGOs except AGO 2 and 10 to substitute AGO1 function in stabilizing mir168. However, the stabilization of mir168 in the triple mutant having *ago1-25* compared to another triple mutant is possibly still maintained with possible other mechanisms in the cell to prevent any more reduction or the possible higher slicing capability of *ago1-25* compared to *ago1-27*. Thus, even though both AGO2 and AGO10 can act as a substitute for the loss of the AGO1 function in this type of mutant, there is no stronger backup other than these two AGOs. Based on this result, the two types of AGO1 mutations may behave differently in mir168 stabilization.

As previously discussed, one of the most important AGO-miRNA connections that possess a crucial role in silencing activity and is tightly controlled on several levels is AGO1-mir168. AGO1 mRNA accumulation is controlled by the mir168, which is normally loaded into AGO1 protein, the product of its target to maintain the homeostasis of AGO1 function (Vaucheret et al. 2006). Previous studies showed a stronger binding preference of mir168 to AGO10 instead of AGO1 based on the mir168 alternative configuration structure, thus showing an antagonistic role between the two (Iki et al. 2018). So AGO10 must also protect miR168 from degradation, and this can explain the strong reduction of mir168 in *ago1-25, 10-1* double mutant and *ago1-27, 10-1* double mutant compared to single *ago1* mutants. Interestingly, ago1, 2-1 double mutants both showed an even stronger reduction. Previous studies described an overlapping role between AGO1 and AGO2 in plantacyanin production (Maunoury and Vaucheret, 2011). Thus, the reduction of mir168 in those double mutants seems to correlate with the overlapping role between the two AGOs. However, the effect of different types of AGO1 mutations on mir168 stabilization might need a further in-depth study since the two *ago1, 2-1* double mutants show different miR168 accumulation. Replica experiments could resolve the question of whether is this due to some kind of environmental condition or it is a biologically relevant result.

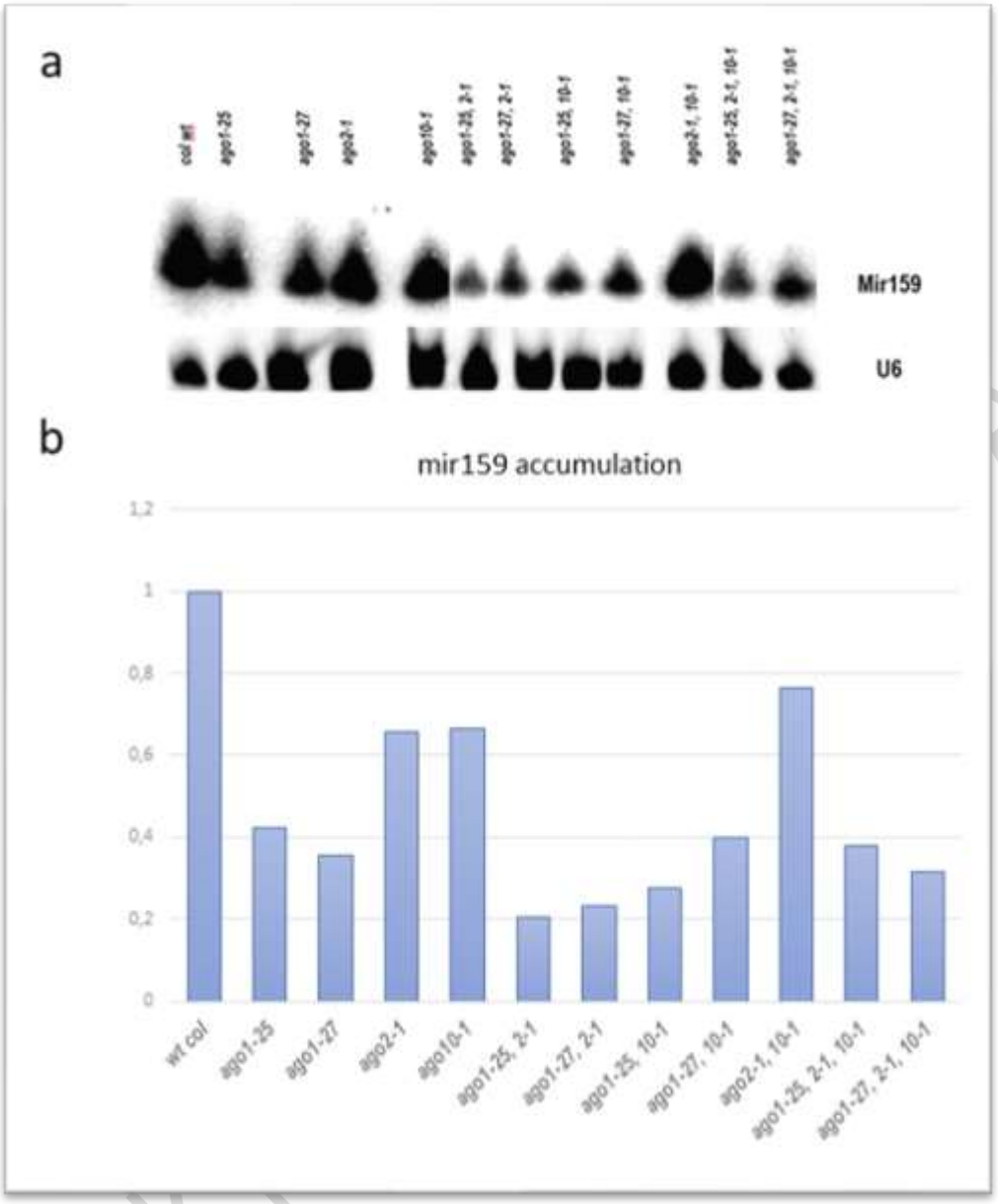

Figure 19. The accumulation of mir159 in mutants
(a) Small RNA northern blot of mutants detecting mir159 accumulation and (b) normalized data of miR159 concerning *A. thaliana. Columbia*. As a loading control, U6 was used.

In agreement with miR168, a reduction of miR159 accumulation was also detected in the case of the mutants compared to *Arabidopsis thaliana Columbia* wild type (**Fig. 19**). This suggests that the absence of a single AGO gene decreases the stability of mir159 and the redundantly acting different AGOs can substitute for the lost AGO function. Based on this result, the reduction of mir159 could reach less than half of the total amount as in *Columbia* wt in all mutants except *ago2-1,10-1*. It can be concluded that all three AGO proteins play a major role in stabilizing mir159, and stabilizing effect of AGOs have a great impact on miR159 accumulation. This is in agreement with the high loading efficiency of this miRNA. As it exists mostly in a loaded form in the cells, most of the miR159 molecules rely on the protection of AGOs against degradation. Indeed, AGO2 and AGO10 could partially act as a substitute for the AGO1 function regarding mir159 stability which can be seen when comparing the single mutants with the double mutants with loss of

AGO2 or AGO10, as a lower-level accumulation of mir159 could only be detected when besides AGO1 another AGOs was also inactivated. However, AGO1 alone plays the most crucial role in this stabilization which can be seen in the accumulation reduction of all the mutant harboring *ago1* mutation while AGO2 and 10 could partially act as a substitute for AGO1 function which can be seen in the case of *ago2-1, 10-1* mutant where the accumulation of mir159 is preserved on a higher level on the absent of AGO1.

As previously described, miR159 is shown to play an important mission in the development of the plant, in seed germination, and the response to the drought. There are several genes encoding mir159. Several studies show the high association of mir159 with AGO1 in plants (Contreras et al. 2012). Although other mechanisms, like over-expression of its precursor RNA or decrease of its degradation, could also influence the accumulation of mir159. The perturbation of mir159 could lead to many defects and lead multiple phenotypical changes. It has been suggested that the mir159 mutant could lead to curled leaves, a change of normal phase development, a delay in flowering time, and defective plant organs, the features that can be seen on the double and triple mutants having *ago1* mutation in this study while over-expression of this miRNA could also lead to the disturbance of flowering time (Archard et al. 2004; Todesco et al. 2010; Rubio et al. 2013).

5.2.4 Monitoring of the *Ago1* mRNA level with semi-quantitative RT-PCR

Ago1 expression is controlled by miR168 through the miRNA pathway. To detect whether the alteration of miR168 accumulation in mutants had a profound effect on the *Ago1* mRNA level, semi-quantitative RT-PCR was carried out. Based on the result of using *Ago1* Forward/Reverse primer pair to detect AGO1 mRNA accumulation, the lower level of AGO1 correlated to some extent with the mir168 accumulation (**Fig. 20**). All *ago1* mutation-containing plants, except for *ago1-27* and *ago2-1* single mutants showed an extreme increase in their Ago1 mRNA level. This increment was higher than expected from the miR168 level, so there might be other factors to trigger it. Probably the loss of AGO1 function evokes also the up-regulation of Ago1 transcription as feedback, so the final mRNA level is due to the composition of less miR168-conveyed cleavage and up-regulated transcription, with whom the plant tries to compensate for the impaired AGO1. It is possible that in *ago1-27* the mutation has no influence that is responsible for this action, even though the two mutations are located in the same domain. As previously mentioned, both of the *ago1* mutations are located in the PIWI domain of the AGO1 amino acid sequence which possessed slicing activity. It can be suggested that a small alteration of the amino acid sequence (in this case, one single substitution of an amino acid in the different parts of the PIWI domain sequence) could affect AGO1-mir168 slicing efficiency toward AGO1 mRNA. From **Fig.20b,** So *ago1-27* mutant line still has a stronger capability of silencing AGO1 mRNA and consequently could trigger less induction of expression. On the other hand, the mutation that occurs in the 759 positions in the *ago1-25* mutant could strongly reduce the cleavage activity of the AGO1 on controlling AGO1 mRNA accumulation. Interestingly, the loss of AGO10 also enhanced the Ago1 mRNA level giving another proof of the coordinated action of the two AGOs. On the other hand, *ago2-1* mutation does not seem to have such an effect as the accumulation of AGO1 mRNA in

the loss of AGO10 and AGO2 together is similar to that of the single *ago10-1*, and the loss of AGO2 function alone does not intensely affect the accumulation of the same mRNA compared to the wild-type.

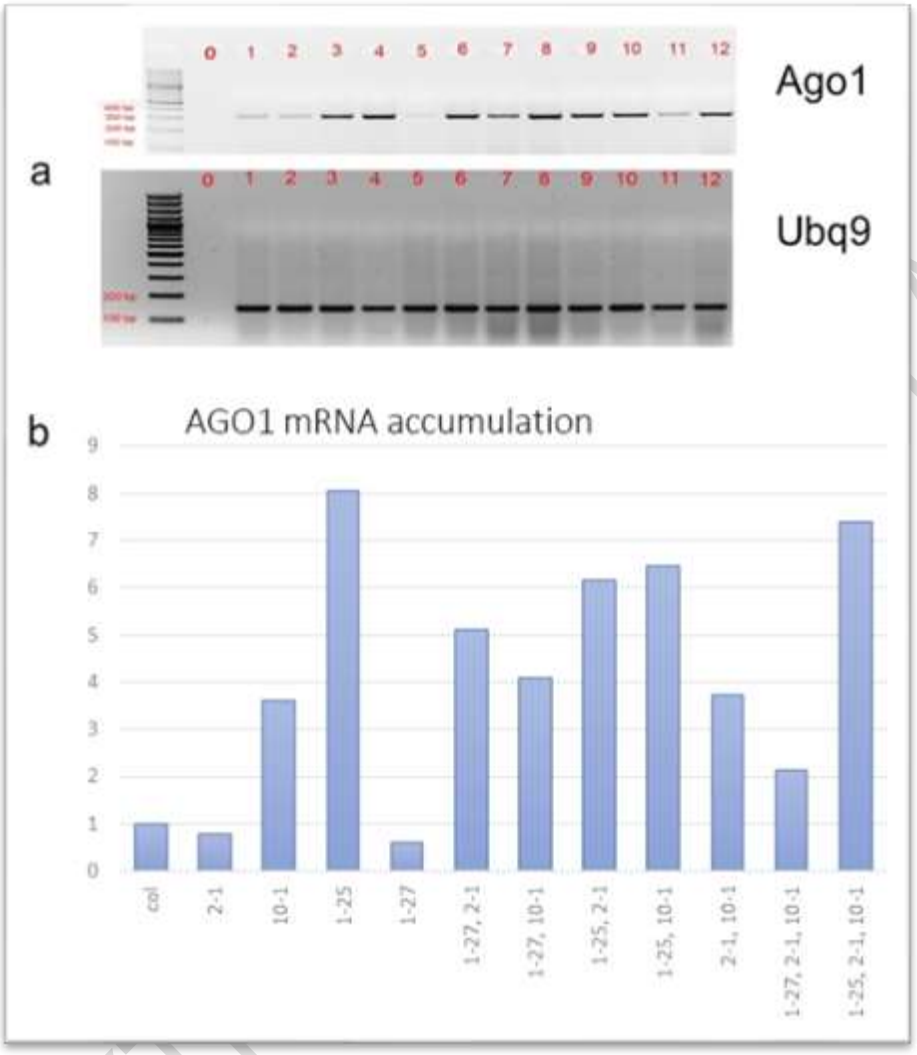

Figure 20. Semi-quantitative RT-PCR to monitor *Ago1* mRNA level in the *ago* mutant plants

The numbers in Figure **(a)** represent (0) negative control, (1) *Arabidopsis* thaliana Columbia wild type, (2) *ago2-1*, (3) *ago10-1* (4*) ago1-25*, (5) *ago1-27* (6) *ago1-27, 2-1* (7) *ago1-27, 10-1* 8) *ago1-25, 2-1* (9) *ago1-25, 10-1* (10) *ago2-1, 10-1* (11) *ago1-27, 2-1, 10-1* (12) *ago1-27, 2-1, 10-1*. GeneRuler 100 bp DNA ladder is used and can be seen in the first lane of the gel **(b)** normalized data relative to A*. thaliana Columbia* in the form of a graph. Data were normalized to Ubq9 expression as a gene of reference.

Taking our previous results into consideration, mir168 accumulation shows a correlation to the expression level of Ago1 mRNA level in all of the mutants except for the *ago2-1* and *ago1-27* single mutant. The high level of AGO1 mRNA accumulation together with the lower level of miR168 in triple mutants shows that in these plants, the miR168 conveyed regulation has a profound role in controlling the Ago1 level. In triple mutants, the loss of AGO2 and AGO10 as a substitute for AGO1 function in mir168 loading might cause the enhancement of the free, unbound mir168 accumulation which became a target for degradation. On the other hand, the presence of either AGO2 or AGO10 in double mutant having *ago1* mutation could to some extent compensate for the mir168 stabilizing effect and control AGO1 expression. For example, as

previously described, an alternative configuration structure of the mir168 duplex could determine the preference for AGO10 and AGO1 toward this miRNA, thus enhancing the complexity of AGO1 mRNA regulation by the mir168 mediated silencing pathway. Thus, possible different results on AGO1 mRNA could be due to this factor, as well (Iki et al. 2018).

5.2.5 Loading efficiency of miRNAs in *ago1-25* and *ago1-25, 2-1, 10-1*

Gel-filtration method based on FPLC can be used to separate large nucleo-protein complexes as RISC. With this method miRNAs loaded or existing in a free pool can be detected simultaneously from the same plant extract. To detect differences between *ago1-25* single and *ago1-25, 2-1, 10-1* triple mutants' gel-filtration was carried out, and fractions were used to detect miR168. There is no HMW detected using mir168 while the accumulation of free mir168 can be seen very strongly in the triple mutant. A low portion of mir168 must be bound to the AGO-RISC complex in *ago1-25* single mutant, but too low to be detected under current circumstances. However, it can be concluded that the presence of more intense unbound mir168 in the triple mutant having the more severe *ago1-25* mutation could have resulted from the suppression of the three AGOs and the less active RISCs (**Fig. 21**).

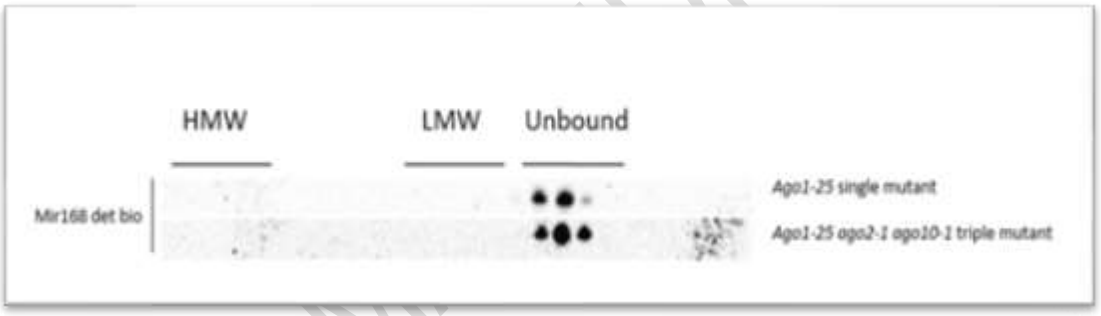

Figure 21. miR168 in FPLC fractions of *ago1-25* and *ago1-25, 2-1, 10-1* mutants

HMW represents High Molecular Weight where the AGO-RISC bounded miRNA can be detected. LMW represents Low Molecular Weight RISC, unbound labeled fractions contain not loaded, free miRNAs. MiR168 was detected with a biotinylated DNA probe.

6. CONCLUSIONS

When *Argonaute* proteins were first described in 1998, AGO1 was identified using the *ago1-1 and ago1-6* mutant of *Arabidopsis thaliana.* This mutant showed similar phenotypic features to our studies like curled-up leaves just like *Argonauta argo* octopus, the origin of its name (Bohmert et al. 1998). As the knock-out mutant had an extremely severe phenotype and was sterile in homozygous form, further studies often used hypomorphic mutants like *ago1-25* or *1-27*. The repressed but not vanished AGO1 function allows the investigation of the role of other AGOs in the developmental processes, as the severity of the phenotype is expected to turn even worse with the removal of other AGOs with presumed substitute working.

In this work, we observed phenotypic changes of *ago1-25*, *ago1-27*, *ago2-1,* and *ago10-1* single mutants and the double and triple combinations of them. All investigations were carried out on plant materials carefully genotyped for bearing the respective mutations in homozygous form. As expected from the literature, all *ago1* mutation-containing plants displayed the typical serrated, curled leaves with long stems. But the intensity of the distortion of organs differed amongst single, double, and triple mutants. The single mutant plants were darker green and had a stunted appearance, with fewer and smaller leaves and fewer and less productive flowers and siliques compared to the wild type. A defect can also be seen in the speed of plant development. Consequently, as mutants having the *ago1* mutation show a lower speed of development, the life span of these plants was far longer than wildtype and other AGO defective plants. Moreover, extreme growth anomalies with undefined organs and unstructured symmetrical growth, including inflorescence stem, flower, and silique could be seen on triple mutants in the late developmental phase. The other single mutants show only mild changes from the wild type. The *ago2-1* mutation caused a slight delay in flowering and a lighter green of the leaves, but these differences are hardly detectable, and the final appearance of the fully developed plant is almost indistinguishable from the wild type. Likewise, the *ago10-1* mutation generated also a mild phenotype hardly differing from the wild type. These plants are slightly stunted, have smaller rosettes and a bit of curled leaf, and their inflorescences are slightly more compact than a wild type. Their double mutant, *ago2-1, 10-1* had also mild phenotypical changes as small developmental delay, shorter internodes in inflorescences, and shorter siliques, suggesting a not essential role in miRNA pathway in the presence of functional AGO1. In contrary with this, *ago1, 2* and *ago1, 10* double and *ago1, 2, 10* triple mutants showed more pronounced defects both in vegetative and generative organ development compared to *ago1* hypomorphic mutants suggesting to have substitute roles in miRNA pathway for these AGOs. Indeed, based on the observation analysis in this research, the AGO1 function is extremely crucial for the development of plants and thus, some regulation pathways might be affected by the disturbance of the AGO1 function, leading to the alteration of some organ's development and function.

SHOOTMERISTMLESS (STM) protein accumulation on regulating shoot apical meristem is controlled by AGO1 and PINHEAD (PNH) redundantly in the embryo where its expression is controlled by miR164 (Barton & Porthig, 1993). Even though this miRNA has not been analyzed in this study, there is a possibility

for the abundance of this miRNA to be disturbed in the case of the suppressed AGO1. Moreover, AGO1 is needed to trigger LEAFY (LFY) into full expression on managing flowering transition and meristem identity (Parcy et al. 1998). APETALA1(APE1) is a gene that negatively regulates AGL1342870/*FRUITFULL(FUL)* expression to control leaf and fruit development, which is strongly reduced in one type of AGO1 mutant (Kidner & Martienssen; 2004). All of them are controlled by mir156 as the study found (Yamaguchi & Abe, 2012). It is also interesting that the upwardly curled leaves that particularly can be found on a triple mutant with *ago1-25* mutation are also recorded in CURLYLEAF-1(CLF-1) mutant (Re et al. 2019). CLF plays an important role in the repression of the homeotic gene, Agamous (AG), the KNOX gene, and APETALA3 (APE3) in leaves (Katz et al., 2004). Homeotic genes such as AG determine the identity of floral organs and control by terminating FM (Floral Meristem) while KNOX genes like STM, KNOTTED-LIKE (KNAT)1-2, and 6 have a role for shoot apical meristem (SAM), and carpel development (Gomez-Mena et al. 2005; Scofield et al. 2007). All in all, disturbances in the miRNA pathway due to the loss of the functional AGOs cause alterations in the level of transcriptional factors defining the vegetative and generative development of plants. And with the loss of that AGOs, which can serve as partial substitutes for AGO1 this effect gets worse.

Furthermore, from the abundance analysis of two miRNAs, we presume that AGO2 and AGO10 can partially act as substitutions of AGO1 function through miRNA-conveyed regulation of plant gene expression. The reduction of mir168 accumulation occurred in all of the mutant lines. The suppression of AGO1 alone could extremely decrease the accumulation of mir168. Interestingly, the absence of AGO10 seems to affect the accumulation of this miRNA as well which can be seen from the noticeable reduction in *ago10-1* single mutant compare to Columbia wild-type, suggesting a redundancy function of the two AGOs. In the suppression of AGO10 and AGO2 together, the accumulation of miRNAs seems still maintained at the level where AGO10 alone is being suppressed, supporting the idea that AGO10 is taking part in mir168 stabilization, exclusively. A previous study showed a stronger binding preference for AGO10 for mir168 than AGO1 based on the mir168 alternative configuration structure, thus showing an antagonistic role between the two (Iki et al, 2018). Moreover, AGO2 proves to also partially act as a substitution for the AGO1 function (and even AGO10). In the suppression of AGO1 and AGO10 together in the double mutants, AGO2 could successfully act as substitution by maintaining the mir168 stability at the level of when AGO10 is suppressed alone, supporting the idea of overlapping role between AGO2 and AGO1 (Maunoury and Vaucheret, 2011). Even though both AGO2 and AGO10 can act as a substitute for the loss of the AGO1 function in this type of mutant, there is no stronger backup other than these two AGOs which can be seen on the very extreme mir168 reduction on the triple mutants. Moreover, it can be also concluded that all three AGO proteins play a major role in stabilizing mir159. Based on this result, the reduction of mir159 could reach less than half of the total amount as in *Columbia* wt in all mutants except *ago2-1,10-1*. It can be concluded that all three AGO proteins play a major role in stabilizing mir159. However, we cannot exclude the possibility of the existence of other AGO proteins not included in this research and serve also as a backup for AGO1 function.

Moreover, mir168 accumulation shows a correlation to the expression level of Ago1 mRNA level in all of the triple mutants except for the *ago1-27*. The studies regarding negative feedback regulation between AGO1 mRNA and mir168 have been studied so many years. The increased transcriptional activity of miR168 correlated highly with an elevation of the Ago1 mRNA transcription, and this resulted in the modest accumulation of AGO1 both on mRNA and protein levels as a study found (Erley et al. 2010). However, the lower accumulation of AGO1 mRNA on the *ago1-27* single mutant in this study arise an interesting question of whether the different mutation could affect AGO1 mRNA accumulation stability. Both of the *ago1* mutations are located in the PIWI domain of the AGO1 amino acid sequence which possessed slicing activity. It can be suggested that a small alteration in the amino acid sequence (in this case, one single substitution of amino acid in the different parts of the PIWI domain sequence) could affect AGO1-mir168 slicing efficiency toward AGO1 mRNA. Further analysis is suggested to be conducted to reveal this relationship.

In addition, based on the gel-filtration analysis, the presence of more intense unbound mir168 in the triple mutant having the more severe *ago1-25* mutation can be detected. No detection appears on the high molecular weight fraction. A study reported a poorly miR168 loaded into the RISC complex in Arabidopsis thaliana Columbia (Dalmadi et al. 2019). The lower intensity of unbound mir168 in the ago1 triple mutant and invisible loaded miR168 in both of the mutants could have resulted from the suppression of the three AGOs and the less active RISCs. A further experiment can be generated to see miR168 loading efficiency in other mutants or to analyze other miRNAs in the experimented *ago* mutant.

Further analysis could be also conducted on other AGO proteins in the plant to see any other possible AGO protein acting as a substitute function for a specific AGO. Moreover, AGO expression analysis can be initiated to a bigger picture of how the *ago1* mutation could change the expression level of some other AGOs. Furthermore, the analysis of bounding miRNAs to the AGO-RISC complex using FPLC can be conducted on other miRNAs in some other mutant plants to visualize the activities of these miRNAs in the suppression of AGO1, AGO2, and AGO10.

SUMMARY

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Functional analysis of *ago1*, *ago2*, and *ago10* mutants

MSc in Agricultural Biotechnology

ARGONAUTEs (AGOs) are the protein group that exists in eukaryotic and procaryotic cells having partly distinct and overlapping functions, specifically in gene silencing pathways together with small RNA (sRNA) as the guide. AGOs – sRNA complexes are important to activate silencing activities. sRNA plays a major role in guiding AGOs into specific targets through sequence complementarity. However, individual ago mutants were extensively studied, and there is still small knowledge revealing the possible overlapping functions among AGO proteins. Particularly, to extend our knowledge about other AGOs than AGO1 taking part in miRNA pathway, AGO2 and AGO10 seem to be appropriate candidates. The objective of this research is to analyze the functional redundancy of AGO1, AGO2, and AGO10 1) through phenotypical analysis of single, double, and triple mutants impaired in the respective genes. 2) To investigate the possible substitution or backup function of AGO2 and AGO10 in the miRNA pathway with the analysis of miR168 and miR159 content in the mutant plants. 3)To investigate the expression of AGO1 mRNA as the target of miR168 in the presence of analyzed mutations, and 4) to visualize the loading efficiency of miR168 into AGO-RISC complex in *ago1-25* and *ago1-25, 2-1, 10-1* mutants.

Single mutants were provided by the research group and originated from the NASC seed bank. Double and triple *ago* mutants were produced by the crossing method of *Arabidopsis thaliana.* F1 progenies of the crossing were tested for their heterozygous nature and self-pollinated. Homozygous plants were selected and left to set seeds. Current work used the next generations/ The seeds were surface sterilized with hypochlorite and germinated *in vitro* on MS medium (Duchefa premix) for one week under continuous light and a constant 21 °C. Seedlings were then kept in peat blocks for further 3 weeks under short day (8 hours light) conditions at 21 °C, planted into pots, and placed on long days (16 hours a day). The phenotypic observation was conducted at the indicated time points (3, 5, 11, and 16 weeks old). Direct PCR was conducted to directly detect the existence of mutant in the plant progeny and total RNA extraction was conducted for further miRNA analysis, including Northern Blotting and FPLC.

Based on the observation, all *ago1* mutation-containing plants displayed the typical serrated, curled leaves with long stems. But the intensity of the distortion of organs differed amongst single, double, and triple mutants with low fertility and different life spans. Some major alterations on the generative organs can also be observed in all mutants having *ago1.* Moreover, an extreme defect can be seen on triple mutants in their later developmental phase with undefined organs and unstructured symmetrical growth, including inflorescence stem, flower, and silique. We also presume that AGO2 and AGO10 can partially act as substitutions of AGO1 and function as a backup AGO for mir168 and mir159 through miRNA analysis on the mutant plants. The result of semi-quantitative RT-PCR can also explain the likelihood between AGO1 expression levels on some analyzed plant mutants where it is highly controlled by the disturbance of the mir168 population except *ago1-27* mutant. moreover, gel-filtration analysis toward *ago1-25* single and triple mutants revealed the presence of more intense unbound mir168 in the triple mutant having the more severe *ago1-25* mutation. The invisible loaded miR168 in both of the mutants could be a consequence of the suppression of the AGOs and the less active RISCs.

In conclusion, the suppression or lack of the investigated AGO proteins, especially AGO1 could trigger some alterations in the development of plants while AGO2 and AGO10 could act as substitutes for AGO1 function. The elevation of AGO1 mRNA level in mutants can be due to the up-regulation of transcription and/or lowered down-regulation of it through the decreased miR168. Further in-depth analysis is needed to prove the action of AGO2 and AGO10 in other aspects of the miRNA pathway and to reveal the possible role of other AGOs.

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SUPPLEMENTARY MATERIALS

Table 1. Total RNA amount from nanodrop quantification

No	Plant lines	Total RNA
1	*Arabidopsis Columbia* wildtype	799,1 ng/ul
2	*ago2-1* single mutant	950,6 ng/ul
3	*ago10-1* single mutant	376,4 ng/ul
4	*ago1-27* single mutant,	1131,1 ng/ul
5	*ago1-25 single mutant*	1384,9 ng/ul
6	*ago1-25 ago10-1* double mutant	1153,5 ng/ul
7	*ago1-27 ago10-1* double mutant	464,8 ng/ul
8	*ago1-27 ago2-1* double mutant	708,3 ng/ul
9	*ago1-25 ago2-1* double mutant	1486,6 ng/ul
10	*ago2-1 ago10-1 double mutant*	1133,7 ng/ul
11	*ago1-25 ago2-1 ago10-1* triple mutants	1384,9 ng/ul
12	*ago1-27 ago2-1 ago10-1* triple mutants	1284,0 ng/ul

Table 2. Primers and probes

No	Primers / probes	Sequence	Usage on experiment
1	AGO1_F	ATGGTGAGAAAGAGAAGAACGGATG	Sq RT PCR
2	AGO1_R	TCAGCAGTAGAACATGACACGCTTC	Sq RT PCR
3	Ubq9_F	F: GTGCTGAGAGATGCGGATTG	Sq RT PCR
4	Ubq9_R	R: CCTCTCCTCCTCCAACAGTC	Sq RT PCR
5	LBb1_SALK	*GCGTGGACCGCTTGCTGCAACT*	Mutant identification
6	AGO2-1_F	TCTAGAGCCACCATGGAGAGAGGTGGT TATCG	Mutant identification
7	AGO2-1_R	GGATCCTCAGACGAAGAACATAACATTC TC	Mutant identification
8	AGO10_LP	AGGTGGCAATCAAGTTTGTTG	Mutant identification
9	AGO10_RP	AATTTTGCATGCCTACATTGG	Mutant identification
10	Mir159	TAGAGCTCCCTTCAATCCAAA	Probe on total RNA analysis
11	Mir168	TTCCCGACCTGCACCAAGC	Probe on total RNA analysis

DECLARATION

on authenticity and public assess of final master's thesis

Student's name:	Bhakti Mahendra Jaya
Student's Neptun ID:	BS6N4Q
Title of the document:	Functional analysis of *ago1*, *ago2*, and *ago10* mutants
Year of publication:	2023
Department:	Plant Biotechnology

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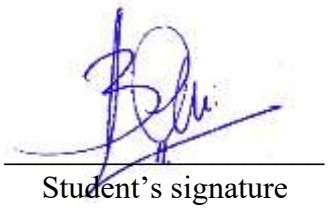

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STATEMENT ON CONSULTATION PRACTICES

As a supervisor of Bhakti Mahendra Jaya (BS6N4Q) I here declare that the final master's thesis has been reviewed by me, the student was informed about the requirements of literary sources management and its legal and ethical rules.

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