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Facultad de Ciencias Biológicas.
Programa de Doctorado en Ciencias Biológicas
Mención Genética Molecular y Microbiología

UNRAVELLING THE ROLE OF ARGONAUTE1 PHOSPHORYLATION IN THE NITRATE RESPONSE OF *Arabidopsis thaliana*

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Santiago, 4 de marzo de 2020

A mi familia, quienes sin titubeos han impulsado mis pasos, erráticos e inseguros, hacia el incierto e infinito aprendizaje.

“It is our choices, Harry, that show what we truly are, far more than our abilities”

- JK Rowling,

ACKNOWLEDGEMENT

This thesis represents the culmination of a process that involves exquisite experiences, learning and impressive human beings. Thanks to the funding given by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) 1141097; Instituto Milenio iBIO-Iniciativa Científica Milenio-MINECON; and Ph.D. fellowship from Comisión Nacional de Investigación Científica y Tecnológica (CONICYT) 21140874.

First, I express my sincere gratitude to my thesis supervisor, Dr. Rodrigo Gutierrez, for the continued support from the beginning to the end of this process. His motivation and confidence proved to be invaluable within all these years. His advice and guide were crucial in my training as a scientist and as a person. I will always be grateful for all the opportunities he gave me.

I would like to thank the people from the Molecular Genetics and Microbiology Department at the Biological Sciences Faculty for the continued support since I was accepted until this instance in the doctoral program. I would also like to thank my thesis committee members Dr. Xavier Jordana, Dr. Patricio Arce, Dr. José O'brien and Dr. Lorena Norambuena for their time, reviews, and for trusting this project.

As wise people say, a good team needs the right people, the right spirit, and a couple inside jokes. Infinite thanks to the research group, PSBlitos, for being the perfect team. It was five years

of sharing with brilliant minds and friends. I would like to especially thank Andrea and Cristofer. Andrea, as the main responsible for this project to come to light, thanks for your time, for the reviews and your goodwill. Cris, thank you for putting up with me as a thesis tutor, teacher and I hope, a trustworthy person. To all the PSBL team, both current and former members: Karem, Mely, Grace, Ale, Tomasín, Seba, Vale, Eleo, Susana, Jony, Sole, Pancha, Ariel, Or, Vivi, JM, Gaby, and many others, thank you for the time invested, for listening and for sharing all your wisdom unconditionally.

I must sincerely thank Dr. Xuemei Chen and her team at the University of California, Riverside, for allowing me to learn in the first person, as first-level research is done. This experience gave me solid skills that not only made me develop as a scientist but as a person.

To the people with whom I started this personal project. Without you all, I could not consider these years as the best of my life. Leslie, thank for your unconditional friendship, brilliant and for showing me what it is not to be afraid. Valito, your honesty, and pragmatism are an inspiration. To Anibal, Herni, Nano, Gise, Jorge, Pancho, and Ire, thank you for the joy and friendship.

I feel great gratitude to the friends who have always been there and who joined along the way. I am grateful to life for putting me in the right place with the right people. I want to especially thank Vale and Chirri for the wine and love that has always accompanied our friendship.

My family deserves much more than a few lines of thanks, I want it to be clear. Infinite thanks to the people who make my world. Thank you, Madre, Gilou, Papá, and my lovely siblings. I could never imagine going through this challenge without the large number of human beings that cushion all my falls, supported me in every decision, and encouraged me to do my best in every moment. I love you all. Atte., una agradecida Isa

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LIST OF ABREVIATIONS

°C	Degree Celsius
^{32}P	Phosphorus-32
%	Percentage
µm	Micrometer
µL	Microliter
bp	Base pair
DIC	Differential Interference Contrast
cm	Centimeter
cDNA	complementary DNA
CDS	Coding sequence
dATP	Deoxyadenosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Doxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
g	Number of times the gravitational force

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hour
HCl	Hydrochloric acid
INRA	Institut National de la Recherche Agronomique
KCl	Potassium chloride
KNO_3	Potassium nitrate
LB	Lysogeny broth
M	Molar
$MgCLl_2$	Magnesium chloride
mL	Milliliter
mM	Millimolar
mm	Millimeter
NaCl	Sodium chloride
nt	Nucleotide
NP-40	Nonyl phenoxypolyethoxylethanol
N-terminal	NH_2-terminal, amino-terminal
PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RNA	Ribonucleic acid
RNase	Ribonuclease H
RT-q-PCR	Reverse transcription polymerase chain reaction
sRNA	small RNA

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBST	TBS and Tween 20
T-DNA	Transfer DNA
Tm	Melting temperature
TRIS	2-Amino-2-(hydroxymethyl)propane-1,3-diol
W/V	Weight per volume

1. RESUMEN

ARGONAUTA 1 (AGO1) es parte esencial del complejo de silenciamiento RISC (inglés de RNA-induced silencing complex), jugando un rol protagonista como endonucleasa cuya actividad controla la expresión génica tanto a nivel transcripcional como post-transcripcional. El presente trabajo surge a partir de la observación en que nitrato altera el patrón de fosforilación de AGO1 en raíces de plantas de *Arabidopsis thaliana*. Nitrato, además de su rol como nutriente, es una molécula que actúa como una potente señal, gatillando una cascada de señalización que controla la expresión de cientos de genes. Si bien, la fosforilación detectada ha sido observada en otros fosfo-proteomas tanto en *Arabidopsis* como otras especies, a la fecha, no contamos con antecedentes que profundicen sobre el rol que cumpliría esta modificación en la función o actividad de AGO1 en plantas. Por otro lado, a nuestro haber existe incontable evidencia que demuestra el rol de AGO1 como una proteína esencial para la respuesta a estrés tanto biótico como abiótico, en la fertilidad y el completo desarrollo de las plantas. A partir de estos antecedentes, nos preguntamos: ¿Cuál es el rol de esta fosforilación sobre la función de esta crucial proteína? Con el fin de hacer frente a esta pregunta, evaluamos procesos del desarrollo y la respuesta molecular de plantas cuyo sitio de fosforilación ha sido mutado. Para ello, generamos plantas cuya proteína AGO1 posee una alanina (A, fosfo-nula) o un ácido aspártico (D, fosfo-mímica) en reemplazo de la serina fosforilable. Evaluamos el impacto de modificar el patrón de fosforilación de AGO1 sobre el crecimiento, incluyendo como variable en el medio diversas concentraciones de nitrato. Nuestros resultados apuntan a que la fosforilación de AGO1 es necesaria para que las plantas completen un ciclo de vida óptimo. Hemos observado que impedir la fosforilación de AGO1 (fosfo-nula) afecta negativamente el desarrollo y fertilidad de las plantas, produciendo fenotipos que abarcan desde un menor tamaño de plántulas, hasta una reducción en el número de semillas. Al analizar los posibles mecanismos causantes de estos fenotipos, determinamos que ni la acumulación ni la carga de los miRNAs sobre AGO1 fueron afectadas. Sin embargo, la abundancia de transcritos de cientos de genes se vio alterada cuando ambas variables fueron evaluadas: la mutación del sitio de fosforilación y la concentración de nitrato en el medio. Tomando en cuenta que la función de AGO1 abarca prácticamente todos los niveles de la regulación génica, pero que la actividad endonucleasa determina el silenciamiento post-transcripcional y, por tanto, la abundancia de transcritos de blancos de miRNAs, pudimos observar que, si bien la actividad *slicer* puede verse alterada, no se anula completamente. Esta y otras observaciones sustentan el hecho de que impedir que ocurra la fosforilación de AGO1 no significa una alteración sustancial del desarrollo sino más bien un conjunto de fenotipos que dan cuenta del efecto pleiotrópico de AGO1, tanto en condiciones normales como bajo diferente disponibilidad de nitrato. Nuestros resultados

apuntan a que la fosforilación de la proteína AGO1 representa un nuevo mecanismo de regulación de la expresión génica en plantas que, en conjunto con otros, modula la abundancia de los mRNAs y como consecuencia, el desarrollo y la respuesta a nitrato en plantas.

En consideración de las múltiples, esenciales y emergentes funciones que abarca la actividad de AGO1 en plantas, dilucidar cómo esta fosforilación y otras modificaciones modulan eventos tales como la localización subcelular y la interacción con otros componentes celulares, es fundamental para comprender el rol de la regulación post-traduccional en la actividad del controlador central de la expresión génica, AGO1.

2. ABSTRACT

ARGONAUTE1 (AGO1) is a key component of the RNA-induced silencing complex (RISC) and has an essential role in transcriptional and post-transcriptional control of gene expression in plants. This work was motivated by the observation that nitrate alters phosphorylation of AGO1 in *A. thaliana* roots. Although this phosphorylation has been identified in several independent phospho-proteome studies in *Arabidopsis*, and a comparable phosphorylation site has been found in rice, there are no reports describing the impact of this phosphorylation on AGO1 function. On the other hand, to our credit, there is countless evidence that demonstrates the role of AGO1 as an essential protein to control gene expression in several biological processes involved in stress responses, fertility and development of plants. From this background, we asked: What is the role of this phosphorylation on the function of this crucial protein? In order to address the role of AGO1 phosphorylation for control of gene expression and its impact on plant development and nitrate responses, we evaluated morphological and molecular phenotypes of AGO1 phospho-null and phospho-mimic mutants plants subjected to various nitrate conditions. Both phosphor-null or phospho-mimic mutants partly complemented *ago1* null mutant lines and exhibited distinct developmental phenotypes. Our results suggest that AGO1 phospho-serine is necessary for plants to complete normally their life cycle. We observed that preventing AGO1 phosphorylation negatively affects plant development and fertility, producing phenotypes that range from a smaller seedling size, to a reduction in the number of seeds. By analyzing the possible mechanisms that cause these phenotypes, we determined that neither the accumulation nor the AGO1-bounded miRNAs were affected. However, the abundance of transcripts for hundreds of genes was altered when both variables were evaluated: the phosphorylation site mutation and the nitrate concentration in the medium. *In vitro* slicing assays showed AGO1 slicer activity may be altered by the Serine 1001 phosphorylation. AGO1 endonuclease activity is crucial for post-transcriptional gene silencing and the abundance of miRNAs target transcripts. Our results support the fact that phosphorylation of AGO1 affects post-transcriptional control of several transcripts with impact on a number of processes. Our results also indicate phosphorylation of AGO1 protein represents a new mechanism for control of transcript abundance in plants that can impact developmental and environmental responses in plants.

Keywords: ARGONAUTE1, AGO1, *Arabidopsis*, miRNAs, development, nitrogen, nitrate, phosphorylation.

3. INTRODUCTION

3.1.1 Plant Small RNAs: Machinery and mode of action

As sessile organisms, plants are constantly challenged by environmental adverse conditions. To cope with all kinds of external and internal changes, plants have developed sophisticated ways to regulate all layers of gene expression. Transcriptional, post-transcriptional, and translational reprogramming are essential steps to set upon protective and adaptive mechanisms to ensure survival.

Small RNAs (sRNAs) are short non-coding RNAs (21-24 nt) that regulate all biological processes through (*1*) transcriptional gene silencing (TGS) mediated by sRNA-directed DNA methylation (Mette et al., 2000; Zilberman et al., 2001; Law and Jacobsen, 2010; He et al., 2011; Creasey et al., 2014; Dolata et al., 2016; Liu et al., 2018; Ma and Zhang, 2018); (*2*) post-transcriptional gene silencing (PTGS), achieved by endonucleolytic cleavage ("slicing") of mRNA target (Baumberger and Baulcombe, 2005; Baulcombe, 2014; Borges and Martienssen, 2015; Ma and Zhang, 2018) and/or by (*3*) translational inhibition (Baumberger and Baulcombe, 2005; Brodersen et al., 2008; Li et al., 2013; Rogers and Chen, 2013; Fang and Qi, 2016). The small but powerful single-stranded microRNAs (miRNAs) and small interfering RNAs (siRNAs) molecules are responsible for such important tasks: to regulate and coordinate gene expression in every organ

and across plant life. The action of both, miRNAs and siRNAs, requires the interaction with ARGONAUTE (AGO) proteins to form the active RNA-induced silencing complex (RISC) (Baumberger and Baulcombe, 2005; Meister, 2013). In plants, the biological role of AGO proteins in PTGS and TGS has been well established (Fang and Qi, 2016). The *Arabidopsis* genome encodes a total of 10 AGO family members, among which AGO1 is the most studied because of its essential role in plant growth, development, and response to diverse stimuli (Morel et al., 2002; Takeda et al., 2008; Wang et al., 2011).

The core biochemical properties of AGO1 protein (116 kDa) resides in four domains common to all AGO proteins: a variable N-terminal domain, a Piwi-Argonaute-Zwille (PAZ) domain, a domain in the middle of the primary structure (MID), and a carboxyl-terminus (C-terminus) PIWI (abb. of P-element Induced WImpy testis in Drosophila) domain (Hutvagner and Simard, 2008; Poulsen et al., 2013). sRNA binding involves the MID and PAZ domains, in which the 5'phosphate and the 3'end are bound by the MID and PAZ domains, respectively (Ma et al., 2004; Poulsen et al., 2013). The PIWI domain adopts a folded structure like RNase-H enzymes which is responsible for the endonuclease activity of *Arabidopsis* AGO1 (Baumberger and Baulcombe, 2005) (FIG. 1A).

Loading of sRNAs into AGO1 protein marks the formation of functional RISC. Plant AGO-sRNA complexes can act through different modes to silence DNA or RNA and exert their biological role. The main modes of action of plant AGO1 are:

(1) Endonucleolytic cleavage: The PIWI domain of AGO1 uses RNAse H-like activity to cleave highly sequence complementarity target RNAs. AGO1 uses slicer activity for at least three distinct purposes *in vivo*: (*a*) Degrading of mRNA of many miRNA targets. mRNA cleavage guided by a 21 nt miRNA in association with AGO1 results in decapped and deadenylated cleavage

fragments called 3’ and 5’ cleavage fragments, respectively, which are subject to rapid degradation (Vaucheret et al., 2004; Martinho et al., 2015; Arribas-Hernández et al., 2016a). (*b*) Generation of cleavage fragments that serve as templates with well-defined 5’– or 3’ -ends for RNA dependent RNA polymerases (RDRPs), transcripts that are cleaved by AGO1 in association with 22 nt miRNAs are not degraded becoming substrates for RDR6 and, resulting in the biogenesis of secondary siRNAs along the length of the cleaved transcripts (Arribas-Hernández et al., 2016b). And (*c*) cleavage of passenger strands in siRNA duplexes to facilitate strand separation (Arribas-Hernández et al., 2016b).

(2) Translational repression: In *Arabidopsis*, AGO1-mediated translational repression occurs in the endoplasmic reticulum and requires almost perfect sequence complementarity (Li et al., 2013). This explains why some *Arabidopsis* mutants are impaired in miRNA-mediated gene repression at protein but not at mRNA levels.

(3) Transactivation: Recent evidence suggests that, besides its role above-described in the cytoplasm, AGO1 is involved in the co-transcriptional regulation of specific *MIRNA* genes in the nucleus in response to environmental stimuli such as salt stress (Dolata et al., 2016). Moreover, a recent publication shows that nuclear AGO1, loaded with 21-nt sRNAs, specifically binds to chromatin and promotes gene expression in response to a variety of environmental cues (Liu et al., 2018).

Then, factors such as AGO1 mutation, post-translational modifications (PTMs), target RNA structure, and presence of interacting proteins might alter AGO1 functions in a variety of ways that will have consequences in the biological functions controlled by small RNAs.

3.1.2. Biological roles of ARGONATE1

AGO proteins are integral players in all described sRNA-mediated regulatory pathways. Loss of *AGO1* expression severely impairs plant development (Bohmert et al., 1998; Vaucheret et al., 2004; Baumberger and Baulcombe, 2005). Phenotypes related to *ago1* loss of function varies from the dwarf and sterile null alleles to fertile hypomorphic alleles that cause less severe developmental defects (Morel et al., 2002; Kidner and Martienssen, 2004; Yang et al., 2006). It's been described *ago1* alleles showed decreased seed germination (Bohmert et al., 1998; Earley et al., 2010), narrow and thickened cotyledons and, dark green and serrate rosette leaves (Bohmert et al., 1998; Kidner and Martienssen, 2004). *Ago1* alleles also show axillary and shoot apical meristem (SAM) defects (Lynn et al., 1999; Kidner and Martienssen, 2005), leaf polarity (Kidner and Martienssen, 2004) and altered timing of developmental phase transition (Morel et al., 2002; Smith et al., 2009).

Besides developmental traits, AGO1 is important for plant virus defense (reviewed in Carbonell and Carrington, 2015), stress response (Earley et al., 2010; Li et al., 2012b), and small RNA biogenesis (Dolata et al., 2016).

To function effectively and to coordinate all the developmental processes and responses that plants execute during their life cycle, events such as the expression pattern, modifications, and cellular localization of AGO1 must be finely regulated.

3.1.3. Plant ARGONAUTE1 activity is highly regulated

In accordance with its central role, it is not surprising that the expression and activity of AGO1 are subject to rigorous and continuous regulation throughout plant life. A process known as AGO1-catalyzed mRNA cleavage negatively regulates AGO1 by miR168 and by itself (Mallory

and Vaucheret, 2009). In the self-regulation pathway, the transcriptional co-regulation of *MIR168* and *AGO1* and, post-transcriptional maintenance of miR168 by AGO1 are essential to ensure efficient RISC loading and activity (Rhoades et al., 2002; Vaucheret et al., 2004; Vaucheret et al., 2006). AGO10, a member of the AGO1 clade, control AGO1 expression by translationally repressing mRNA AGO1 through miR168 activity (Ji et al., 2011).

Additionally, both the cleavage and translational repression activities exercised by AGO1-sRNAs complexes are influenced by AGO1-interacting partners. Known plant AGO1 interactors are CYCLOPHILIN 40 (CYP40), HEAT SHOCK PROTEIN 90 (HSP90), and TRANSPORTIN 1 (TRN1) (Smith et al., 2009; Iki et al., 2010; Iki et al., 2011; Cui et al., 2016). AGO1 loss of function partners impacts severely on sRNA-directed gene silencing as was observed by Iki et al. (2011) and Cui et al. (2016) in their work. This means that is not only necessary to control *AGO1* transcription or mRNA levels to correctly exert miRNA activity, but the coordination with other protein players might employ a mechanistic impact on its global function.

In addition to PTGS, regulation by PTMs is also exerted on AGO1 stabilization and RISC function in the context of plant-virus interactions (Chiu et al., 2010; Alvarado and Scholthof, 2012). AGO1 is subjected to a proteolytic regulation by viral P0 or endogenous F-box protein FBW2. P0 protein command the host SCF-complex to ubiquitinate AGO1 and then degrade it by autophagy (Derrien et al., 2012). Another virus modulator is P25 suppressor, which guides ubiquitinated AGO1 to the proteasome (Chiu et al., 2010). These ways to control AGO1 stabilization act independently, which indicates that there are specific pathways to control AGO1 abundance and action in plants.

3.1.4 PTMs as mechanistic factors for functional ARGONAUTE proteins

PTMs are crucial to modulate the functional diversity of the proteome and particularly AGO proteins. As it was mentioned earlier, AGO PTM has been mainly described in the context of plant-virus interactions (Alvarado and Scholthof, 2012; Yang et al., 2016). The mechanistic behind AGO1 degradation is still poorly understood and, with it, the role of PTMs in this process. On the other hand, much more is known regarding mammal AGO PTMs. Animal AGO proteins undergo PTMs on multiple residues being phosphorylation the best characterized (Lopez-Orozco et al., 2015; Gebert and MacRae, 2019).

Phosphorylation is the most widespread type of PTM and plays a major role in plant signal transduction and metabolism by altering protein activities, protein interactions, stabilization, or protein localization (Mithoe and Menke, 2011; Schönberg and Baginsky, 2012; van Wijk et al., 2014; Silva-Sanchez et al., 2015). Serine (Ser), threonine (Thr), or tyrosine (Tyr) residues are covalently modified by kinases, which transfer a phosphoryl group from ATP to the hydroxyl group of their target proteins (Champion et al., 2004; Nakagami et al., 2010). Animal AGO2, the orthologous for plant AGO1, undergo phosphorylation in many residues. Two phospho-sites (p-sites) are located in the linker that connects the PAZ and MID domains. For instance, Ser387 phosphorylation increases miRNA activity (Franklin et al., 2016) while Tyr393 phosphorylation reduces miRNA-AGO2 association, altering miRNA activity (Shen et al., 2013; Yang et al., 2014). Phosphorylation within the MID domain prevents miRNA loading (Rüdel et al., 2011), and a phosphorylation cycle of a cluster of serine in the PIWI domain regulates the release and processing of target mRNAs (Golden et al., 2017; Quévillon Huberdeau et al., 2017). Although a lot of research about the role played by different phosphorylation sites in the animal AGO2 has

been done, to date, no report states this type of modification as relevant in the function and/or homeostasis of plant AGO1.

Many phospho-proteome studies have detected *Arabidopsis* AGO1-derived phospho-peptides corresponding to one serine residue located at the protein C-terminus (Sugiyama et al., 2008; Jones et al., 2009; Reiland et al., 2009; Nakagami et al., 2010; Wang et al., 2013a; Wang et al., 2013b; Zhang et al., 2013; van Wijk et al., 2014; Roitinger et al., 2015; Somers et al., 2015; Rayapuram et al., 2017). Though, to date, the role of this or any other phosphorylatable residue of plant AGO1 is unknown.

It is clear that AGO proteins, specially AGO1, have a crucial role across plant development and the responses to several environmental cues that plant faces. A lot of evidence shows that each of AGO1 functions and actions are highly controlled by transcriptional, post-transcriptional, and translational regulation. Due to its broad action spectrum, it would be interesting to study AGO1 phosphorylation under one or more external stimuli in plants.

3.1.5 Nitrate as a nutritional signal.

Nitrate is the main source of inorganic nitrogen (N) in agricultural and natural lands (Crawford et al., 2002). Given the fluctuating N soil concentration plants must face, sophisticated adaptive mechanisms act to respond to exogenous N availability. Besides its role as a nutrient, nitrate triggers local and systemic signaling pathways that modulate gene expression, metabolism, physiology, and developmental processes in plants (Bouguyon et al., 2015; Vidal et al., 2015; Poitout et al., 2018). N supply has direct effects on leaf growth (Cai et al., 2017), root system architecture (Tian et al., 2008; Vidal et al., 2010; Sun et al., 2017), flowering time (Marín et al., 2011; Liu et al., 2013; Yuan et al., 2016; Lin and Tsay, 2017), among others developmental traits.

Thus, during the last decades, active research has been done focused on physiological and genetical plant responses to this nutrient. With the advances of -omics technologies, thousands of nitrate-responsive genes have been identified in *Arabidopsis* (Gutiérrez et al., 2007; Gifford et al., 2008; Vidal et al., 2015; Varala et al., 2018) revealing several transcriptional factors that mediate gene expression, especially in roots (Alvarez et al., 2014; Guan et al., 2017; Zhao et al., 2018). Other molecular players that have been described as key components in the nitrate signaling pathway include the nitrate transporter and sensor NRT1.1/NPF6.3 (Bouguyon et al., 2015; Rashid et al., 2018), several protein kinases (Liu et al., 2017), and the ion calcium (Ca^{2+}) (Riveras et al., 2015; Rashid et al., 2018). However, less is known regarding the post-translational regulation, and especially, the modulation of PTMs by the nitrate signaling pathways. Therefore, the following question arises: how the plant phospho-proteome dynamic is modulated by nitrate in *Arabidopsis*? So, in the short term, plants can adapt signaling pathways and, then, their metabolism and growth, according to N conditions.

In our research group, Vega et al. (in process) found more than 200 phospho-proteins with different phosphorylation patterns in response to nitrate. As early as at 5 min post-treatment, kinases and transcription factors (TFs) were differentially phosphorylated, while, at 20 min, proteins involved in transport, protein binding and RNA metabolism were identified. Interestingly, AGO1 phosphorylation at the serine residue located at the 1001 position (Ser1001) showed reduced phospho-peptide levels after nitrate treatments (5 mM KNO_3) when compared with control treatments (KCl 5 mM) or no-treatment conditions (T0). This p-site is right next to the end of the highly conserved PIWI domain, which is responsible for the catalytic RNase-H activity of AGO1 (Baumberger and Baulcombe, 2005). This is not surprising given that most experimentally described p-sites (71%) are situated out of functionally conserved domains (Riaño-pachón et al.,

2010). Moreover, none of the almost 60 *ago1* alleles that have been described have genetic changes localized out of the C-boundary of the PIWI domain. Nevertheless, close to one-third of the alleles affect some features related to the PIWI activity. Even though this phosphorylatable residue had been identified but not characterized in plants, we wondered if it was relevant for (1) the molecular and cellular function of AGO1 and (2) the phenotypic and molecular response to nitrate availability of *Arabidopsis thaliana*. The study of this modification connects miRNA-machinery activity and the coordination of the balance between plant development and environmental cues.

miRNA prominence in the development and responses of *Arabidopsis* to external stimuli is clear. Plant adjustment to nitrogen availability is a complex process that includes deep physiological and biochemical modifications controlled by hundreds of genes and metabolic pathways. Numerous studies have been conducted to get knowledge about the role of nitrate-responsive miRNAs in different plant species. Such effort has uncovered important miRNAs such as miR393, miR169, and miR528 whose function directly impacts the response of *Arabidopsis* and maize roots to the nitrate stimulus (Vidal et al., 2010; Trevisan et al., 2012).

According to miRNA and nitrate relevance, we propose that AGO1 phosphorylation is a PTM that alters its function and the nitrate response as part of the mechanism that controls the physiological and metabolic adjustment required to cope with one or several external changes. In order to test this hypothesis, we set to put the aim to determine the degree of alteration of AGO1 function when the Ser1001 was modified. In short, we characterized the phenotyping responses of the mutant in the Ser1001 residue. Then, we determined the impact on plant responses to different nitrate availability and, we got insights about the functional and molecular mechanism(s) involved in the AGO1 phosphorylation.

The investigation of the morphological and molecular consequences of mutating the AGO1 Ser1001 residue were studied by replacing it for an alanine (S1001A) or an aspartic acid (S1001D) reasoning that the presence of a non-charged A or a negative charge from the acidic residue of D would mimic either an unphosphorylated or a phosphorylated residue, respectively. The present work showed that phospho-mutants did exhibit significant but not severe changes in shoot and root growth under normal or contrasting nitrate conditions. Development of flower and seed production, as well as the timing of developmental phase transitions, were also affected. However, miRNA abundance and miRNA association to AGO1 were practically unchanged, while miRNA targets abundance and AGO1 cleavage activity resulted impaired mostly in the plant expressing the unphosphorylated AGO1 protein. This is the first report indicating that the residue Ser1001 of AGO1 is relevant for plant development and, it is probably implicated in target miRNA processing and gene expression silencing. Since this work contributes to understanding the role and the mechanistic function behind AGO1 Ser1001 phosphorylation, we are closer to revealing the posttranslational regulation affecting AGO1 function in *Arabidopsis thaliana.*

4. MATERIALS AND METHODS

4.1 Plant material.

Arabidopsis thaliana plants ecotype Columbia (Col-0 and Col-2) were used for all experiments. The *Arabidopsis* lines *ago1-1 and ago1-27* were generously donated by Dr. Hervé Vaucheret from Institute Jean-Pierre Bourgin, INRA Centre de Versailles-Grignon, France.

Arabidopsis seeds were surface sterilized with 70% ethanol for 7 minutes followed by other 7 min of 50% bleach wash. Finally, seeds were rinsed 4-5 times with sterile deionized water. Seeds were cold stratified at 4°C for 2 days to synchronize germination.

4.2 Growth conditions and treatments.

Plants were grown in plate or soil conditions. Seeds were sowed in plant growth Murashige and Skoog medium (MS) 50% with or without nitrogen supplemented with the required antibiotics and nitrate concentrations.

4.2.1 Contrasting nitrate concentrations treatments.

Arabidopsis seeds were grown in square plates using MS media without nitrogen (M531, Phytotechnology Laboratories, http://www.phytotechlab.com) supplemented with two different

nitrate concentrations: 0.5 or 5 mM KNO_3 (101187, Merck). Both represent limiting and sufficient nitrogen concentration, respectively.

4.2.2 Nitrate treatments

For nitrate treatments, approximately 1500 *Arabidopsis* seeds were grown in 200 mL hydroponic cultures using phytatrays (P1552-100EA from Sigma) and mesh polyamide (6 stm, 250 μm) for two weeks under long-day (16h light/8-h dark) conditions at 22°C in plant growth incubators (Percival Scientific, http://www.percival-scientific.com). MS-modified basal salt media without N was used, supplemented with 0.5 mM ammonium succinate (cat. N. 1.00682, Merck) and 0.1% sucrose (cat. N. 1.07687, Merck). Plants were treated for the indicated period at the beginning of the light cycle on day 15 with 5 mM nitrate potassium (KNO_3), or 5 mM potassium chloride (KCl, cat. N. 104938, Merck) as a control. A period of 2 weeks was chosen for treatments due to this is the earliest time at which no detectable ammonium is found in the media. Hence, no source of nitrogen remains when we perform the experiments to evaluate plant responses to nitrate treatments.

4.3 Analysis of developmental traits.

4.3.1 Shoot, root, and other plant developmental features.

For early analysis, seeds were sown onto square Petri plates arranged horizontally or vertically for shoot area and root length analysis, respectively. Rosette and root image analysis was performed 7, 10, 12 and 14 days after sowing using ImageJ (http://imagej.nih.gov/ij).

Flower development and biomass seed were evaluated in soil-grown plants. The seeds were harvested and weighed at the end of the lifecycle of the plant. Seeds of 10-15 independent plants were used for each replicate.

For the phenotypic analyses of roots treated with nitrate, plant images were acquired using an Epson Perfection V700 photo scanner (Epson, http://www.epson.-com), and primary roots were measured using ImageJ. Initiating and emerging LRs were counted using DIC optics on a Nikon Eclipse 80i microscope, as previously described (Vidal et al., 2010).

4.3.2 Evaluation of developmental phase transition timing.

To measure the developmental phase transitions timing, at least 10 plants of each mutant line and the wild-type control were grown in the growth chamber in individual pots arrayed in a 5 x 8 grid in standard greenhouse flats. Relative humidity was maintained at 60-70%. Plants were watered as needed, usually every 2 to 3 days. Day length was 12 hrs. (neutral day) or 16 hrs. (long day), and daytime and nighttime temperatures were maintained at 22 and 20 °C, respectively. The day when the first pair of true leaf appears marked the heterotrophic to autotrophic transition, while the leaf on where appeared the first abaxial trichome was used as a marker of the transition from autotrophic seedling to juvenile adult (Huijser and Schmid, 2011). Finally, the change to a reproductive adult or flowering time was indicated by the number of rosette leaves when the stem was 1 cm length (Boyes, 2001).

4.4 DNA constructs and plant transformation.

The AGO1 phospho-null (AGO^{S1001A}) and phosphor-mimic (AGO^{S1001D}) DNA constructs were generated by site-directed mutagenesis of *Arabidopsis AGO1* cDNA through Gibson Assembly as described by Gibson et al. (2009) (For details to prepare Gibson assembly mix go to: dx.doi.org/10.17504/protocols.io.hpib5ke). Oligonucleotides used are listed in TABLE N° 1. Oligos S1001A and S1001D contain the codon for the alanine (A) and aspartic acid (D),

respectively. Complete 3153 nucleotides *At*AGO1 CDS were amplified from the cDNA clone C105223 (vector name: pUNI51) by using the oligonucleotides AGO1 F2 and AGO1 R3 and the enzyme Phusion® High-Fidelity DNA polymerase (2U/µL). To obtain the full promotor of *AGO1* the primers AGO1 F1 and AGO1 R2 were used to perform the PCR using genomic *Arabidopsis* DNA as a template. Genomic DNA extraction was made using the CTAB extraction protocol described in Keb-Llanes et al. (2002) with minor modifications (Buffer CTAB (2% (w/v) CTAB, 1% polyvinyl pyrrolidone (PVP),100 mM Tris-HCl (pH 7.5), 1.5 M NaCl, 25 mM EDTA, 0.3% (v/v) β-mercaptoethanol)). Restriction assay and sequencing analysis were used for sequence checking. The DNA constructs AGO1^{S1001S}, AGO1^{S1001A} and AGO1^{S1001D} were used to transform *Rhizobium radiobacter* (*Agrobacterium tumefaciens* GV3101 strain) by electroporation (MicroPulser^{TM} Electroporator, 1652100, Bio-Rad) as described (Mersereau et al., 1990) adding 1 ug of the helped plasmid pSOUP. *Agrobacterium* selection was made in LB medium using the following antibiotics: kanamycin sulfate 50 µg/mL, gentamicin sulfate 25 µg/mL (G1914, Merck), tetracycline hydrochloride 10 µg/mL (T859, Phytotechnology Laboratories) and rifampicin 50 µg/mL (R0146, Duchefa Biochemie) as selection markers.

Phospho-mutant plants were obtained by *Agrobacterium*-mediated *Arabidopsis* transformation following the protocol described by Zhang et al. (2006) with slight modifications (Floral dip infiltration solution: 1.1 g/L MS basal salt, 0.5 g/L MES; adjusted pH 5.7, 5% sucrose, 50 µL/liter Silwet L-77). Transformant selection was followed in 50% MS Basal Salt Mixture (M550, PhytoTechnology Laboratories LLC, Shawnee Mission, KS) supplemented with 0.1% sucrose, 7g/L agar-agar (cat. N. 05040, Merck) and, the antibiotics kanamycin sulfate 50 µg/mL (K4378, Merck) and hygromycin b 15 µg/mL (stock 50 mg/mL in PBS, H370, Phytotechnology Laboratories).

Plants able to grow in presence of both antibiotics were checked using polymerase chain reaction (PCR) to amplify the *AGO1* cDNA insert and, T-DNA insert within the intron 9 of wild type *AGO1* locus from genomic DNA. We designed a forward and reverse set of primers, using the intron 9 sequence of the *AGO1* gene. The combination of forward and reverse I9 primers only generates and 837 bp amplification product when using DNA extracted from wild type plants. Homozygous mutants were selected at T2 generation and propagated for sequential experimental procedures.

TABLE N° 1. List of oligonucleotides used for Gibson assembly and genotyping.

Amplified region	Oligonucleotide name	Sequence (5´→ 3')	Tm (°C)	Length (bp)
Gibson				
Promoter AGO1	F1	tttcatcttcatcttcata**taggaacacaaacctccatcg**	56	2269
	R2	tcttctctttctcaccat**gatgattcctgtgaaaataacacaaccacc**	60	
AGO1 CDS	F2	attttcacaggaatcatc**atggtgagaaagagaagaacggatg**	58	3147
	R3	ccgggcatcttgaggttc**tcagcagtagaacatgacacgctt**	60	
Vector backbone	F3	gtcatgttctactgc**tgagaacctcaagatgcccggcg**	62	5326
	R1	atggaggtttgtgtt**cctatatgaagatgaagatgaaatatttggtg**	56	
Mutagenesis				
AGO1 S1001A	S1001A F	acatggagccagagacagctgacagtggctcaatggctag	71	
	S1001A R	cattgagccactgtcagctgtctctggctccatgtagaat	70	
AGO1 S1001D	S1001D F	acatggagccagagacagatgacagtggctcaatggctag	71	
	S1001D R	cattgagccactgtcatctgtctctggctccatgtaga	69	
Genotyping				
AGO1 intron 9	I9_F	tgtgttgctgcacaggtatg	57	837
	I9_R	gagtctggaagggaagggac	57	
AGO1 Insert	F	cctactgttctggctcagc	56	1090
	R	gttgaaggccagcggtattc	57	

Abbreviations: F: forward, R: reverse

4.5 Immunopurification of AGO1 complex.

For immunopurification of AGO1 complex, 1 gram of frozen *Arabidopsis* seedlings was grinded into fine powder under liquid nitrogen and homogenized in 2 mL of plant extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 4 mM $MgCl_2$, 5 mM DTT (R0861, Thermo Fisher), cOmplete™ EDTA-free protease inhibitor cocktail (4693132001, Sigma, 1 tablet/10 mL buffer), and PhosSTOP™ (4906845001, Sigma, 1 tablet/10 mL buffer) was added. Cell debris was removed by centrifugation at 20,000 g for 20 min three times. Extracts were pre-cleared by incubation with 50 µL of Protein A agarose Dynabeads (cat. N. 10002D, Thermo Fisher Scientific) at 4°C for 60 min. In parallel, 100 µL of Dynabeads were incubated with 10 µL of anti-AGO1 antibody (cat. N. AS09527, Agrisera) rotating at room temperature for 30 min. Then, beads were washed three times (15 min each) in plant extraction buffer containing 2 mM DTT at 4°C. Finally, beads were resuspended into 100 µL of plant extraction buffer and 6 µL were used for the evaluation of immunopurification (IP) efficiency by western blot.

4.6 Protein blot analysis.

Total proteins were extracted from 12-days old seedling or 14 days-old roots growth in hydroponic culture using the plant protein extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 4 mM $MgCl_2$, 5 mM DTT), cOmplete™, EDTA-free protease inhibitor cocktail, and PhosSTOP™. Ground tissue was incubated for 2 hours at 4°C rotating and, centrifugated for 20 minutes at 4°C at 12000 g. Supernatant was transferred to a new tube and quantified by using BCA kit. Protein samples were resolved by SDS-PAGE and electro-transferred to PVDF membrane previously activated with methanol (cat. N. 1.06009.2500, Merck). After 1h

blocking in 1X TBST with 5% non-fat milk, antibody incubations were carried out 1h at room temperature with constant shaking. Primary antibody anti-AGO1 and anti-GAPDH antibodies were used at 1/10000 and 1/5000 dilution, respectively. Then, membranes were washed three to five times in TBST and incubated for 1h in secondary antibody peroxidase-conjugated goat anti-rabbit antibodies. Finally, the membrane was rinsed three to five times with TBST before detection with the Pierce ECL Western blotting detection kit (Thermo Fisher Scientific cat. N. 32106).

4.7 RNA isolation and real-time q-PCR analyses.

RNA was isolated from whole roots and seedling using TRizol® Reagent (cat. N. 15596-018, Invitrogen, Carlsbad, CA, USA). Only for roots, RNA isolation was made in conjunction with the PureLink® Mini Kit to isolate total transcriptome RNA from frozen tissue (ThermoFisher Scientific, https://www.thermofisher.com, Cat. N. 12183025). cDNA synthesis was carried out using the SuperScript® II reverse transcriptase according to the manufacturer's instructions (ThermoFisher Scientific, https://www.thermofisher.com, cat. N. 18064014). cDNA synthesis was performed using random primers (cat. N. C1181, Promega, Madison, USA) and the ImProm-II Reverse Transcriptase kit (cat. N. M314A, Promega, Madison, USA). Then, RT-q-PCR was performed using the Brilliant III Ultra-Fast SYBR® Green QPCR Reagents on a StepOnePlus™ Real Time System (qPCR). The RNA levels were normalized relative to CLATHRIN ADAPTOR COMPLEXES MEDIUM SUBUNIT FAMILY PROTEIN (*CLAT*) (At4g24550).

4.8 Small RNA blot analysis.

Total RNA was isolated from frozen tissue and ground with pestle and mortar in TRIzol® reagent. Small RNAs were separated by 15% denaturing PAGE-Urea gels and transferred onto

Hybond N^+ membrane (GE Healthcare) and cross-linked via EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) cross-linking, as previously described (Pall and Hamilton, 2008). Oligonucleotides complementary to *miR156, miR172, miR390 and miR393* and U6 were labeled by incubation with T4 PNK in the presence of [^{32}P] dATP. The hybridization was performed as described (Wu et al., 2009) and U6 RNA was used as a loading control.

4.9 Small RNA accumulation analysis.

4.9.1 Library preparation.

Twelve-days old *Arabidopsis* seedlings were collected as starting material for small RNA and mRNA high-throughput sequencing. sRNA libraries for sequencing were constructed following Illumina's TruSeq Small RNA protocol (NEBNext® Small RNA Library Prep Set for Illumina). In brief, 3' and 5' adapters were sequentially ligated to the ends of RNA < 40 nt long, fractionated from 20 g from gel-purified total RNA and, reverse transcribed to generate cDNA. The cDNA was amplified (11 cycles of PCR) using a common primer complementary to the 3' adapter, and a 5' primer containing 1 of 48 index sequences. Samples were size selected on a 6% polyacrylamide gel, purified, quantified, and pooled for multiplexed sequencing. The resulting pooled libraries were hybridized to oligonucleotide coated single-read flow cells for cluster generation on-instrument and subsequent sequencing on an Illumina HiSeq 2500 instrument. Fifty sequencing cycles were performed.

4.9.2 Bioinformatic analysis of the sequences.

Raw Illumina sequencing reads were trimmed to remove the adapter sequence (AGATCGGAAGAGCACACGTCT) using Trimmomatic (Bolger et al., 2014). Trimmed reads were aligned against the *A. thaliana* genome sequence TAIR10.26, or the sequences of the 427 *A.*

thaliana mature miRNAs annotated in miRBase v21. Reads perfectly mapped to mature miRNA were aligned using strand-specific alignment. Only full-length perfect matches were selected. Data normalization and the subsequent differential expression (DE) comparison (mutant/Col-2) were performed by DESeq2 tool, (Love et al., 2014) using a significance cut-off of 0.05.

4.10 Gene transcript accumulation analysis.

4.10.1 Libraries preparation and sequencing.

We analyzed 12-old days plants grew on standard conditions, 5 mM or 0.5 mM KNO_3. As a control, wild-type (Col-2) and mutant (AGO1) plants were grown as described. Total RNA was treated with DNAse (TermoFischer, cat. N. EN0771) to eliminate trace DNA contamination. Further processing including sample quality and quantity verification was made on an Agilent Fragment Analyzer™ prior sequencing. Libraries were made using Illumina Truseq stranded mRNA library prep kit and RNA sequencing was carried out by Macrogen Inc. in an Illumina Novaseq 100bp PE platform (Macrogen Inc, Seoul, Korea; http://www.macrogen.com).

4.10.2 Bioinformatic analysis of the sequences

To determine DE genes under contrasting nitrate conditions, we use the DESeq2 package from Bioconductor (Love et al., 2014). The transcript abundance of all expressed genes from each sample was calculated and the median values of these from each sample were used to normalize the gene read counts from RNAseq data (Contreras-López et al., 2018). After normalization, the lowest 25% expressed genes were deleted. Then, we generate two DE genes list by using two methods. The first lit includes genes that are regulated when comparing each library (genotype/condition) against all the other libraries. For the second case, we normalized the expression by subtracting the reads of the wild-type (Col-2) to every other mutant line. Then, a

two-way ANOVA was performed to compare the transcript abundance of each gene within the genotype (G), the growth condition (C = nitrate availability) and those genes whose transcript level depended on both variables (C:G) corresponding to the intersection of the C and G lists. That means we identify those genes regulated by the treatment, those exclusively regulated by the effect of mutation and those whose transcript abundance was altered by a combination of both factors, using Col-2 for normalization but eliminating it as a variable.

4.11 *In vitro* RISC activity assay.

4.11.1 Preparation of ^{32}P-labeled target RNAs.

[^{32}P] UTP-labeled T7 polymerase transcript probes corresponding to *PHAVOLUTA* (*PHV*) and *phv* were generated *in vitro* using the Riboprobe® T7 kit (cat. N. P1440, Promega). The template for *in vitro* transcription was generated by PCR using specific primers for the *PHV* gene (forward: 5'-GCCGGAACAAGTTGAAG-3' and reverse: 5'-GACAGTCACGGAACCAAGATG-3'). T7 promoter sequence (TAATACGACTCACTATAGGG) was added to the 5´end of the forward primer. To generate the RNA template, the reaction shown in TABLE Nº2 was prepared (Qi and Mi, 2010).

4.11.2 Cleavage activity assay.

Cleavage assays were performed mixing immunoprecipitated AGO1 (see 4.5 section) and ^{32}P-labeled *PHV* or *phv in vitro* transcripts (Riboprobe® T7 *In vitro* transcription system, Promega) as follows: Four µL of 5x cleavage buffer, 12 µL of purified AGO1 complex, and 4 µL of labeled RNA (from step 8.11.1). Cleavage products were evaluated after incubation for 120 min at 25-30°C. The reaction was stopped by adding 250 µL od Trizol®, 4 µL of 5 mg/mL glycogen, 50 µL of chloroform and, centrifuged at 15,000g for 5 min. The aqueous phase was transferred to

a new tube, RNA was precipitated by adding 150 µL of isopropyl alcohol and, incubated for 30 min at -20°C. Finally, centrifuged RNA was dried at room temperature for 10 min and dissolved in 20 µL of 2x loading buffer. RNA was resolved by 8% polyacrylamide gel/8 M urea and gel was exposed to a phosphor screen to detect the cleavage products.

TABLE N° 2. Reaction for *in vitro* RNA template synthesis.

Reagent	*Volume (µL)*
5x cleavage buffer	4
DTT, 100 mM	2
RNasin RNase Inhibitor (40u/µL)	1
rATP, 10 mM	1
rCTP, 10 mM	1
rGTP, 1 mM	1
rUTP, 1 mM	1
M7G Cap analog[1], 10 mM	1
UTP[α-32P]-250UC (800 Ci/mmol)[2]	5
T7 RNA polymerase	15-20 u
Template	1
Final Volume	**20**

[1] Cat. N. P1711, Promega
[2] Cat. N. 007X250UCPER, Perkin Elmer
[3] Riboprobe® System-T7, cat. N. P1440, Promega

5. RESULTS

5.1 PART I. Nitrate modulates phosphorylation of the conserved Serine1001 residue of ARGONAUTE1.

5.1.1 Nitrate does not alter ARGONAUTE 1 mRNA or protein abundance.

In order to identify new components involved in nitrate responses in plants, our research group performed a phosphoproteome time-course analysis of *Arabidopsis thaliana* roots in response to nitrate treatments. The results and conclusions obtained in this study are been prepares for publication (Vega A., et al (2019), in preparation). For the present work, it is sufficient to say that nitrate modulates phosphorylation pattern of a number of proteins involved in control of gene expression. Among these proteins, we found reduced AGO1 phospho-peptide levels 20 minutes after nitrate treatments (5 mM KNO_3) as compared to control treatments (KCl 5 mM) or no-treatment conditions (T0) (FIG 1A-B). AGO1 is a key component of the RISC complex which, together with miRNAs, plays an essential role in post-transcriptional control of gene expression. Because of the central relevance of this protein in several biological processes in plants, we decided to study the role of AGO1 phosphorylation in more detail. First, we wished to determine whether nitrate was altering phosphopeptide but neither protein nor mRNA abundance. T do so, we measured total AGO1 mRNA and protein from nitrate-treated *Arabidopsis* roots (FIG 1C-D).

As we can observe in FIG 1, nitrate treatments do not impact AGO1 mRNA or protein levels as compared with control (5 mM KCl) or untreated plants (T0). These results suggest that nitrate might act as an external stimulus affecting AGO1 phosphorylation pattern in the short-term nitrate response. Moreover, considering that phosphorylation is as rapid and transient protein modification, our results opened the question: Is this residue important for plant developmental or molecular responses to nitrate in the short or long-term?

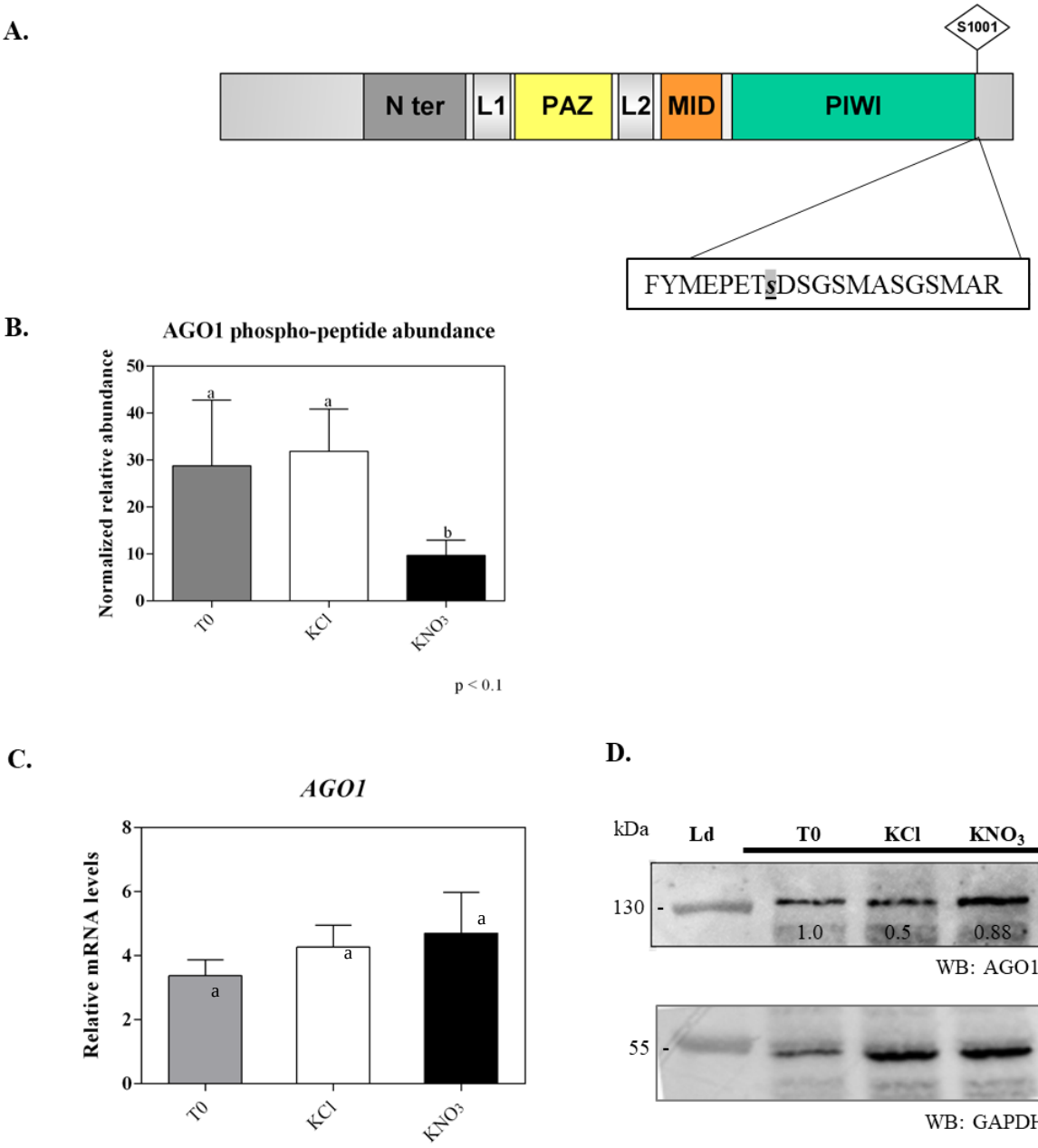
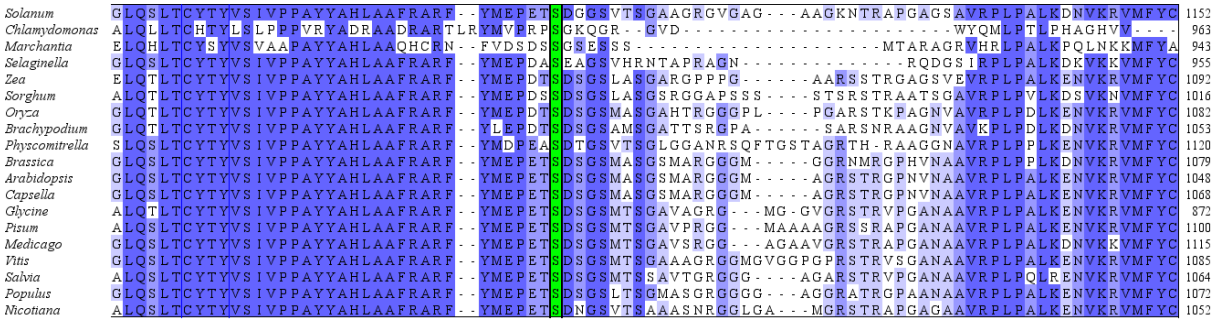


FIGURE 1. Nitrate treatment reduces AGO1 phospho-peptide abundance in *Arabidopsis* roots. A. The core biochemical properties of AGO1 protein resides in four domains: a variable N-terminal, PAZ, MID, and a C-terminus PIWI domain. It is shown the peptide sequence of AGO1 identified by LC-MS/MS. The serine residue within the highlighted sequence corresponds to the phospho-serine detected in *Arabidopsis* roots. **B**. The abundance of AGO1 phospho-peptide detected in phospho-proteome profiling (Vega et al., in prep.). **C – D.** AGO1 mRNA and protein levels after 20 minutes of nitrate treatment of *Arabidopsis* roots. GAPDH was used to normalize AGO1 protein abundance which is shown below the figure D.

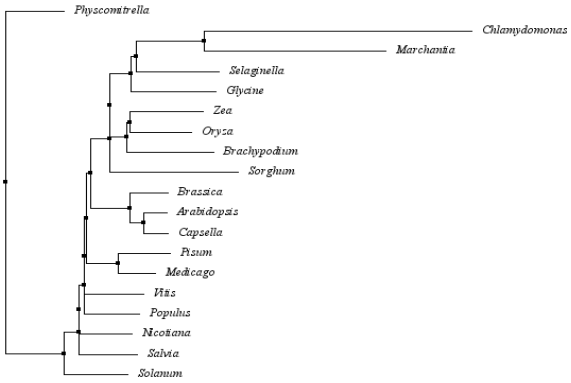
5.1.2 ARGONAUTE 1 Ser1001 residue is conserved across the plant kingdom.

Taking the phosphoproteome analysis data performed by our research group (Vega et al., in prep.), in which AGO1 phosphopeptide presents a differential accumulation in *Arabidopsis* roots, we wondered whether AGO1 Ser1001 residue would be conserved among other plant species or within the *Arabidopsis* AGO family. To answer that, we compared the amino acid sequence of *Arabidopsis* AGO family proteins and AGO1 putative orthologous among plant species. FIG 2 shows sequence alignment in which dark blue means a high conservation degree and green has highlighted the residue of interest. As is shown in FIG 2, although the Ser1001 site is not conserved among the members of the *At*AGO family (except AGO5, which belongs to the same clade as AGO1) nor among the orthologs in mammals or other eukaryotes, within the plant kingdom, this residue is highly conserved in both monocotyledons and dicotyledons (FIG 2). This observation is telling us that the residue Ser1001 has been conserved from bryophytes (i.e *Physcomitrella patents*) to angiosperms (i.e. *Capsella*) and might be particularly relevant for a function that is exclusive to AGO1 in plants.

A.



B.



C.

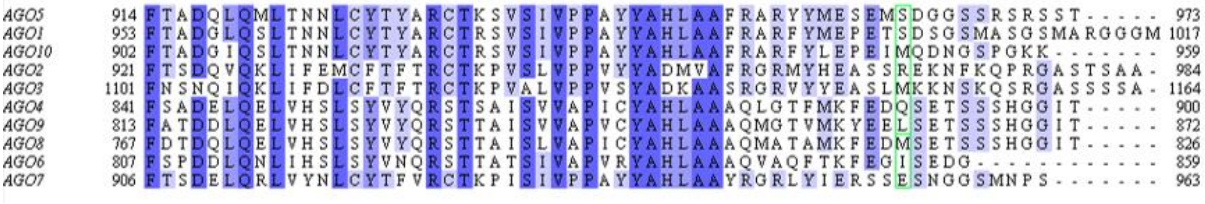


FIGURE 2. Sequence alignment of orthologous AGO1 protein from various species. **A.** Amino acid alignment of AGO1 C-terminus and Ser1001 conservation among plant species. The numbers on the right indicate the amino acid position. The more conservation between amino acid residues the darker the blue background. The residue studied here is shown in a green background. **B**. Phylogenetic relationship of AGO1 with other plant orthologous proteins. The Neighbour Joining tree was made with the Jalview tool using the whole protein sequence alignment of AGO1 proteins from other species. **C.** Carboxyl-terminus amino acid alignment of *Arabidopsis* AGO protein family. The green square indicates the position of Ser1001.

5.2 PART II. Arabidopsis ARGONAUTE1 Ser1001 residue is important for plant growth and development.

5.2.1 AGO1 phospho-mutants present growth and morphological phenotypes

ARGONAUTE1 has a crucial biological role in miRNA activity, and it is involved in practically all plant growth and developmental stages (Bohmert et al., 1998; Vaucheret et al., 2004; Baumberger and Baulcombe, 2005; Carbonell et al., 2012). Although several phospho-proteome studies have detected Ser1001 phosphorylation site in plants, no evidence revealing its impact on AGO1 function and, therefore, on plant development has emerged yet. Mutant alleles in the gene that codes for AGO1 produce varied morphological phenotypes, ranging from dwarf and infertile to imperceptible defects in plant development (Morel et al., 2002; Kidner and Martienssen, 2004; Vaucheret et al., 2004). Several studies point that the place where the mutation is located determines the degree to which the molecular function of the protein is affected. Considering the key role played by sRNAs in plant development, we wanted to investigate the relevance of AGO1 phosphorylation in plant growth, morphological characteristics, and temporality of developmental phase transitions of $AGO1^{S1001A}$ and $AGO1^{S1001D}$ plants.

To determine whether Ser1001 residue is relevant for the correct function of AGO1, first, mutant plants expressing $AGO1^{S1001S}$ (wild type), $AGO1^{S1001A}$, and $AGO1^{S1001D}$ were generated. Through site-directed mutagenesis, the amino acid serine was replaced by an alanine (Ala, A) by changing the TCA codon to GCT. On the other hand, for the generation of $AGO1^{S1001D}$ the mutation of the TCA codon by GAT replaced the serine residue for aspartic acid (Asp, D). Mutants and wild-type *AGO1* cDNA were coupled to 2260 bp of the *AGO1* promoter by Gibson assembly (used primers are listed in TABLE N° 1). These constructs were employed to stably transform

ago1-1 allele in which a T-DNA insertion in the ninth intron within the *AGO1* locus annulate its function leading infertility and a greatly disturbed body architecture.

In contrast to the *ago1-1* null allele, which shows a stunted and severely impaired growth phenotype, dark green hypocotyl and unexpanded pointed cotyledons (Bohmert et al., 1998) (FIG 3A), the expression of complemented wild-type AGO1 ($AGO1^{S1001S}$) fully restores a normal phenotype into the *ago1-1* null mutant background. Following a normal embryo development, the $AGO1^{S1001A}$ and $AGO1^{S1001D}$ cDNA complementation also restore, but partially, the wild-type phenotype of *ago1-1* null plants allowing plants to fully complete their development (FIG 3). Mutant AGO1 protein and mRNA levels were as abundant as the endogenous AGO1 in wild-type plants (FIG 3).

Boyes et al., (Boyes, 2001) defined *Arabidopsis* growth stages both in plate and soil conditions. In plates, leaf development allows the identification of *principal growth stage 1* in which 6 days after sowing (DAS) cotyledons are fully opened (stage 1.0) while 10 DAS shows two rosette leaves bigger than 1 mm (stage 1.02). We have observed that the phospho-defective ($AGO1^{S1001A}$) is smaller than wild-type and the phospho-mimic mutant (FIG 3A, C). To evaluate whether there is an early or late growth arrestment, we registered shoot area development of 4, 6, 8, 10, and 12 DAS plants. It is worth to mention that the germination rate did not show any difference between mutants and wild-type plants, either growing in soil or MS plates (data not shown). Both *ago1-1* complemented mutants ($AGO1^{S1001S}$, $AGO1^{S1001A}$, and $AGO1^{S1001D}$) and wild type (Col-2) plants displayed no difference in the timing of 1.0 and 1.02 developmental stage (FIG 3C). However, $AGO1^{S1001A}$ developed a smaller rosette area and primary root length than wild type plants growing in MS 50% or in soil (FIG 3A, C). Moreover, $AGO1^{S1001D}$ mutant exhibits normal growth, and a longer primary root length than wild-type plants (FIG 3D). These

observations agreed with other reports in which *ago1* hypomorphic alleles show smaller shoot size but longer primary root, probably due to a slow rate of organ production (Morel et al., 2002; Vaucheret et al., 2004; Sorin et al., 2005). Our results indicate that, at early developmental stages, AGO1 phosphorylation is relevant for the proper growth of *Arabidopsis*. After the observation that the phospho-mutants rescue AGO1 functions but displaying developmental phenotypes, we suggest that the phosphorylation of AGO1 affects both shoot and root growth at different growth stages.

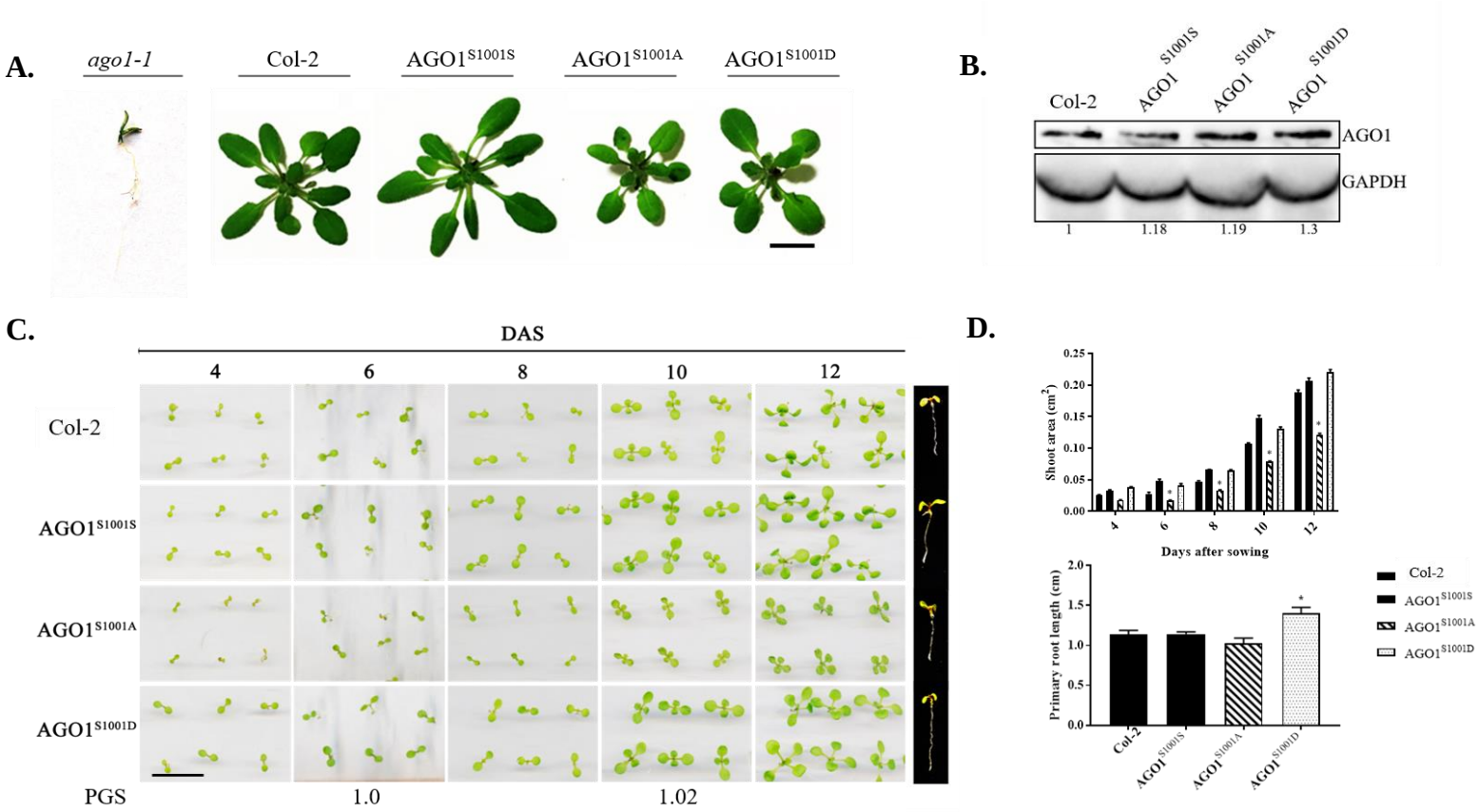


FIGURE 3. Phospho-serine mutants rescue AGO1 function displaying mild developmental phenotypes. A. Three-weeks old plants grown under neutral day conditions (black line = 1cm). **B**. AGO1 protein expression in Ser1001 mutants and wild-type plants. **C.** A representative set of 4, 6, 8, 10, and, 12-days old wild-type and AGO1 mutant plants grown in plate supplemented with 0.1 % sucrose and 50% MS are shown in the left (PGS = principal growth stage). To show primary root length, seedlings grown in vertical plates are shown in the right. **D.** Top: shoot area quantification for each genotype. Bottom: primary root length quantification.Three biological replicates were analyzed. For shoot area quantification > 60 plants were measured for each replicate. For primary root length, 12 plants per replicate were evaluated. An asterisk indicates the significant differences among the genotypes using two-way ANOVA, $p < 0.0001$.

5.2.2 The transition from autotrophic to vegetative adult is altered in the phospho-null AGO1 mutant.

The life cycle of plants undergoes critical developmental transitions that depend on genetic programs controlled by both environmental signals such as photoperiod, temperature, and nutrient status, and internal cues such as plant age, TFs, and miRNAs (Wu et al., 2009; Huijser and Schmid, 2011; Chen et al., 2018). After seed germination, plants initiate heterotrophic metabolism. Then, plants advance from the juvenile seedling to the adult stage of vegetative growth, finally entering the reproductive (flowering) phase. Different morphological changes characterize each of these transitions. It has been demonstrated in detail that miRNAs play central roles that control the timing of developmental transitions (Li and Zhang, 2016). Therefore, we analyzed whether the AGO1 Ser1001 residue was relevant during developmental phase changes. First, we examined the heterotrophic to autotrophic transition by registering the day when the first true leaf pair appears (>1 mm) for AGO1^{S1001A}, AGO1^{S1001D}, *ago1-27* hypomorphic allele (Morel et al., 2002), and wild-type Col-2 and AGO1^{S1001S} plants growing under long and neutral day conditions (FIG 4, upper and bottom panel, respectively). We chose *ago1-27* because homozygous mutants initiate flowering later than wild-type and are fertile. Also, they produce almost 60% of the number of seeds than wild-type (Morel et al., 2002).

According to other studies, our analysis showed a delay in the initiation of new leaf primordia for *ago1-27* mutants (PTGS defective) (Wang et al., 2008). On the contrary, AGO1^{S1001A}, and AGO1^{S1001D} true leaves developed normal and timely as observed for wild-type plants which are consistent with what was given in the previous section, where early development is not affected at a glance (FIG 4, left panel).

c

Finally, we evaluated the transition to the reproductive stage. It was previously reported that *ago1-27* allele initiates flowering 7 to 12 days after wild type plants (Morel et al., 2002). Our analysis showed that *ago1-27* allele did not shoot by the time the rest of the genotypes have flowered, as it was reported. On the other hand, the $AGO1^{S1001A}$ mutant displayed a slight but not significative delay in flowering time under neutral day conditions. Moreover, we observed that phospho-mimetic mutant flowered earlier than phosphor-null (FIG 4 right panel), and for a longer period, completing flower production after wild type plants.

These results show that AGO1 phosphorylation would affect the transition from juvenile to adult. Specifically, this developmental stage is accelerated when Ser1001 phosphorylation is inhibited. The previous observations are consistent with the idea that vegetative phase change and reproductive development are coordinated by the components of the machinery of small RNA activity in *Arabidopsis.*

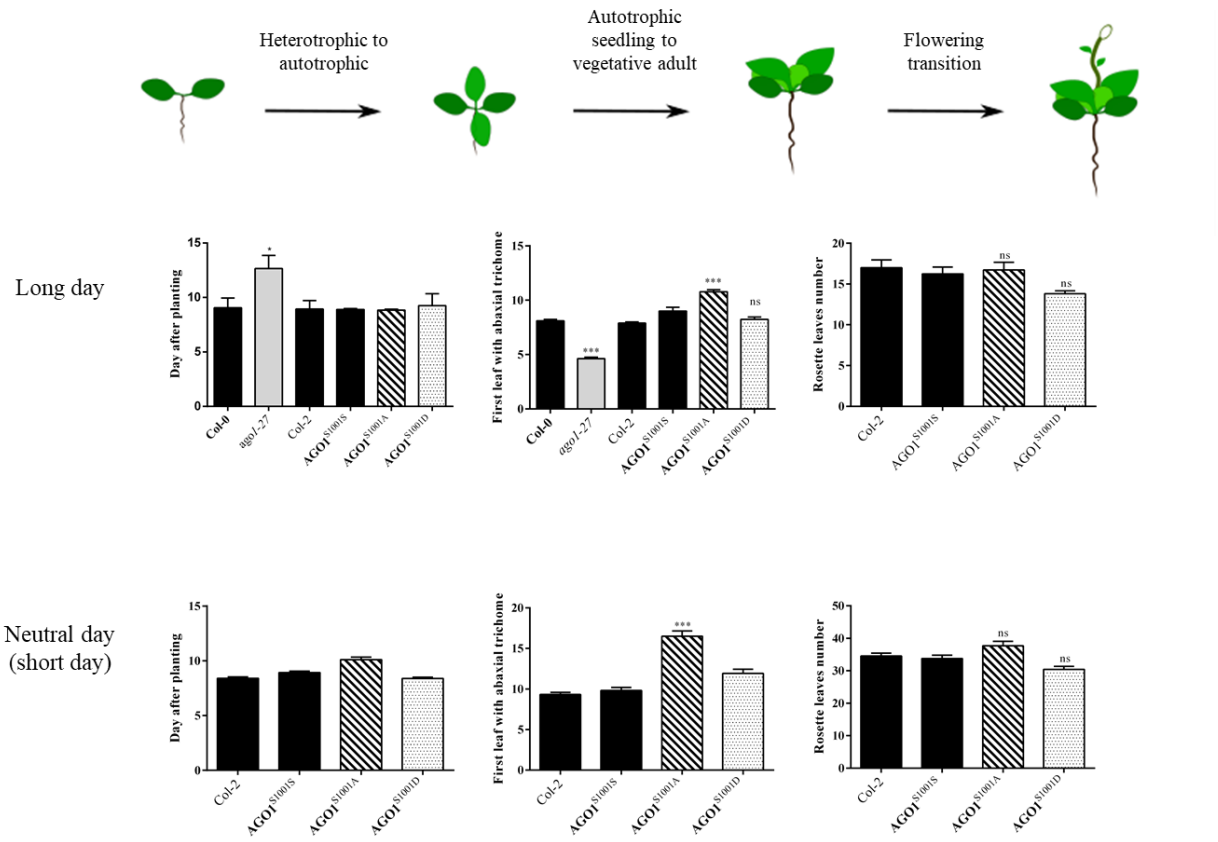


FIGURE 4. Regulation of developmental phase transitions in *Arabidopsis* by AGO1 phosphorylation. Three successive developmental phases were examined for the AGO1 phospho-mutants, *ago1-27* and the corresponding wild-type (Col-2 and Col-0, respectively). Phospho-mutants do not show significative differences in heterotrophic to autotrophic transition (left panel) and flowering time (right panel). However, the phospho-null mutant shows a delay in the transition from autotrophic seedling to vegetative adult (middle panel). Long day (upper panel) and neutral day (bottom panel) conditions were evaluated. *n* = three biological replicates, 10-12 plants each replicate. One-way ANOVA, $\alpha = 0.05$.

To go more deeply into the molecular causes behind the observed phenotype, we evaluated the transcript accumulation of key regulators of the *Arabidopsis* development. miRNAs have roles in floral transition and flower development. For example, the age-dependent transition from vegetative to reproductive growth is regulated by *MIR156*, which gradually decreases with age resulting in elevated expression of its targets, *SQUAMOSA PROMOTER BINDING-LIKE* (*SPL*) TFs which are in turn upstream activators of floral meristem identity genes, *LEAFY* (*LFY)* and *APETLA1* (*AP1)*, as well as *MIR172*. miR172 targets *APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF)* family and its closely related paralogs genes and thereby controls floral transition and whorl-specific activation of floral homeotic genes (Xu et al., 2016). In order to evaluate the involvement of these regulators on AGO1 phospho-mutants development, the transcript levels of *SPL3, SPL9, AP1, AP2, SCHLAFMÜTZE* (*SMZ*), *SCHNARCHZAPFEN* (*SNZ*), and *TARGET OF EAT 1* (*TOE1)* were measured 7, 14 and 21 days after sowing (FIG 5), representing cotyledons fully opened, early and late pre-flowering, respectively. As is shown in FIG 5, most of the evaluated genes displayed a normal behavior compared to wild type. However, *SPL3* and *SPL9* genes showed reduced levels of mRNA which could be explaining at least part of the delay on the juvenile-to-adult transition of AGO1^{S1001A} mutant. Interestingly, we observed a similar SPL3 and SPL9 transcript level in the AGO1^{S1001A} and AGO1^{S1001D} even when phenotype was different between mutants. Due to plant development and phase transitions are controlled by many other pathways, genetic and physiological factors, the integration of TFs, DNA binding proteins, miRNA regulation, and AGO1 activity control into a system-level regulatory level is needed to understand the role of AGO1 phosphorylation in plant development and response to stimuli. Although there are still many pieces to be assembled regarding the function of AGO1

phosphorylation during *Arabidopsis* developmental phase transitions, it would be worthwhile to investigate the expression of these genes during later developmental stages as well as in specific organs such as flowers, meristem, and leaves.

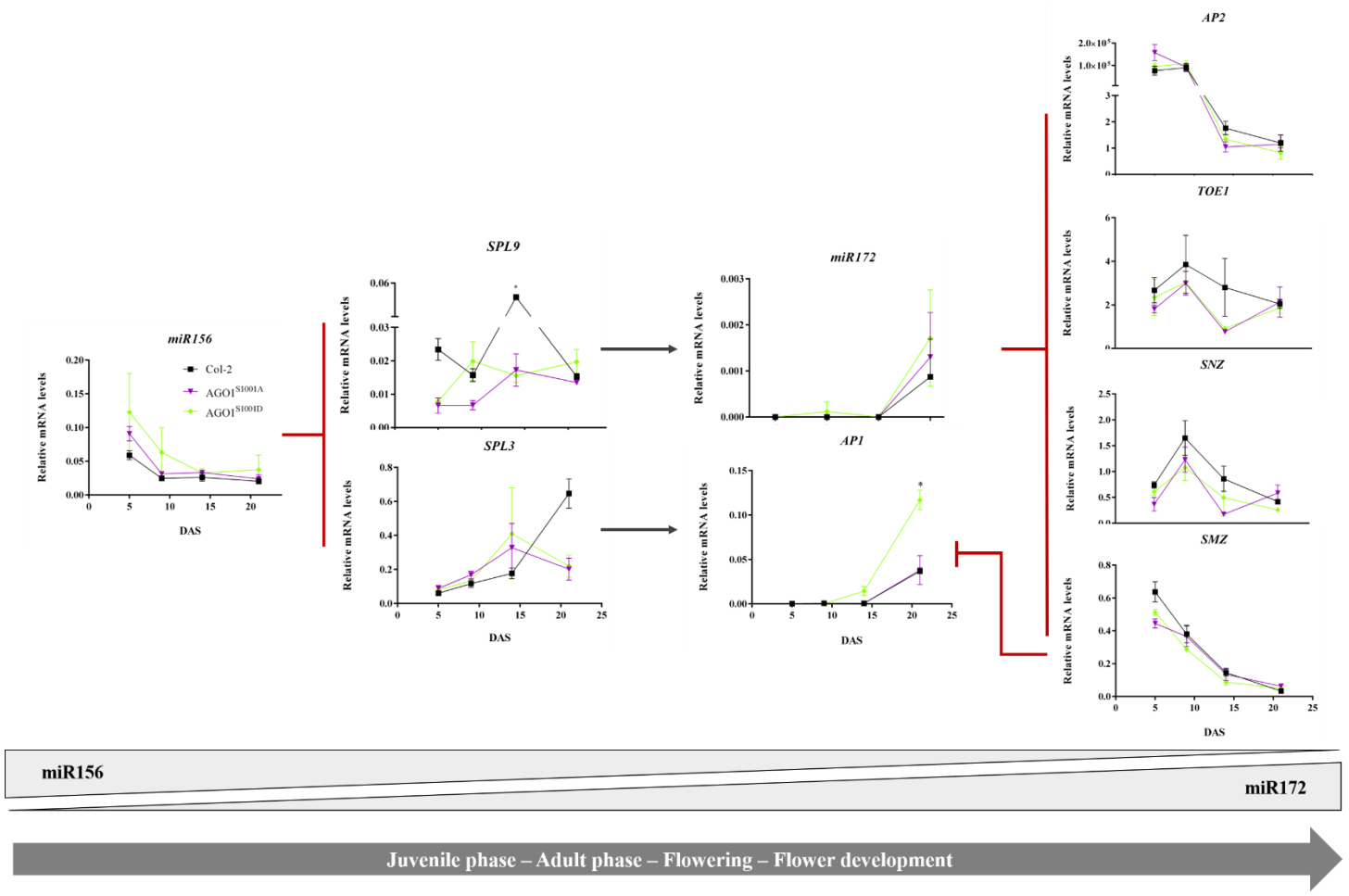


FIGURE 5. Time-course RT-qPCR analysis of the sequential action of miR156 and miR172 targets genes in the AGO1 phospho-mutants during early developmental stages. Plots are ordered according to the developmental phase control from left (early development) to right (late development). The indicated genes were normalized to *CLAT*. Shown are means and SE of the mean for n = 3. DAS = Days After Sowing. Red lines indicate inhibitory relation, black arrows indicate the induction of gene expression. Violet lane: AGO1^{S1001A}, Green lane: AGO1^{S1001D}

5.2.3 AGO1 Ser1001 mutations induce flower defects and reduced fertility.

While the previous results suggest that AGO1-Ser1001 phosphorylation impacts to some extent, early plant growth, and vegetative development, we wondered if AGO1 phosphorylation controlling the late stages of plant development and plant productivity. When reaching adulthood (after 8 weeks of life), AGO1^{S1001D} mutant developed filamentous cauline leaves, abnormal flowers, most of them sterile and, a great number of short and aberrant siliques (FIG 6). AGO1^{S1001D} present smaller bud size and a delay in the visibility of petals than wild type. Moreover, during stage E. 14, AGO1^{S1001D} shows a loss of maximum petal opening, irregular angles, and sloping carpel (FIG 6A). Importantly, AGO1^{S1001D} siliques contain less than half seed than wild type plants (FIG 6B-C). On the other hand, AGO1^{S1001A} mutant displayed a smaller number of abnormal siliques and produced more seeds than AGO1^{S1001D} but less than wild types. It is worth to mention that the AGO1^{S1001D}, which presents a slight acceleration in the transition to the reproductive phase, continues the production of flowers for a longer period than the wild type (data not show).

The abnormal flower maturation and decreased seed production in AGO1^{S1001D} mutant indicate that the phosphorylation of *At*AGO1 might be controlling at least one step in these developmental processes. Previous studies have shown that null alleles of AGO1 display more severe phenotypes than AGO1^{S1001A} and AGO1^{S1001D} described here (Lynn et al., 1999; Vaucheret et al., 2004; Vaucheret et al., 2006), indicating moderate fertility of the mutants presented here.

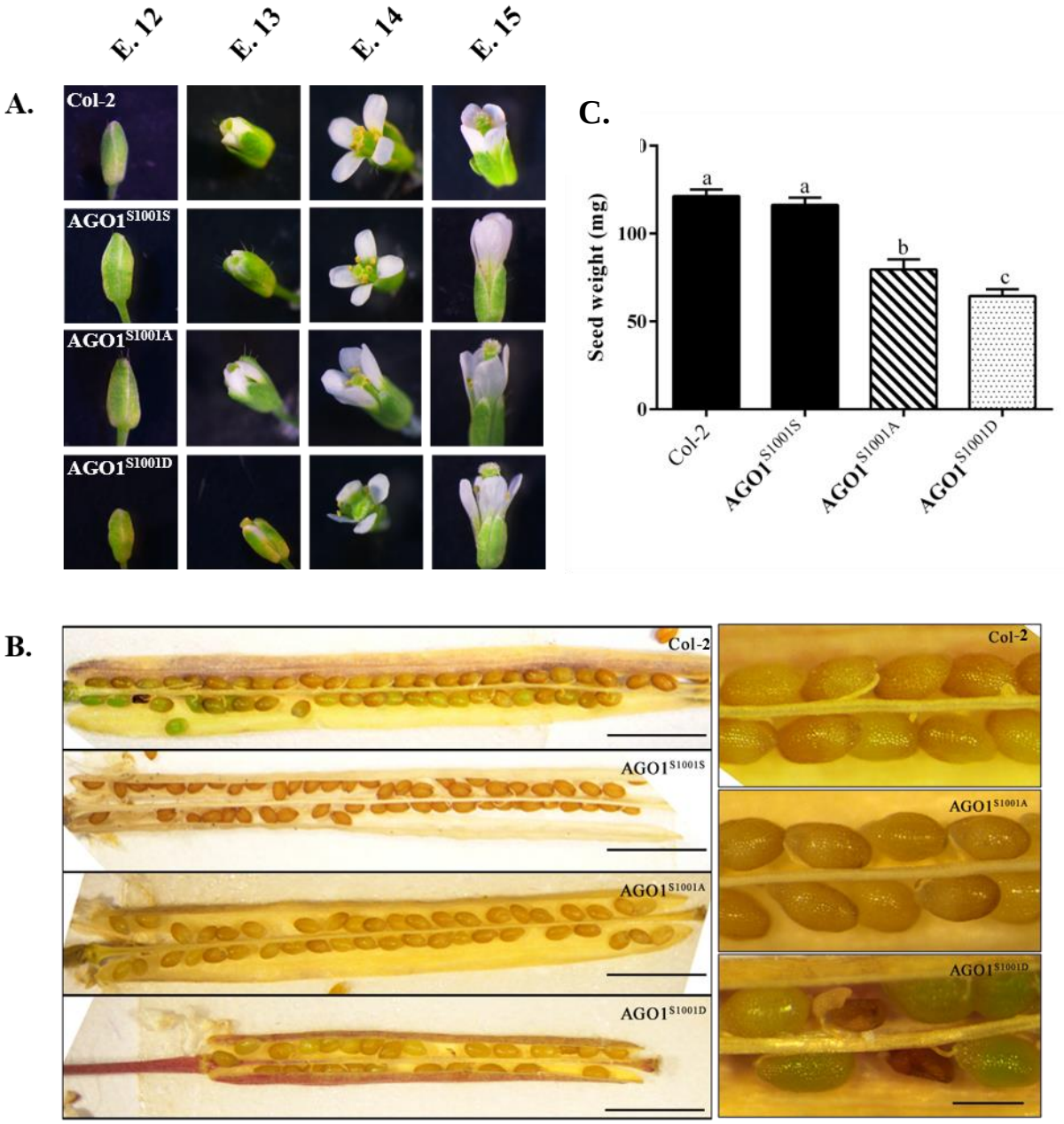


FIGURE 6. AGO1 S1001 phosphorylation impacts the growth of reproductive organs and leads to a reduction in seed content in *Arabidopsis thaliana*. **A**. Wild-type (Col-2) and AGO1^{S1001A} and AGO1^{S1001D} flowers at different stages of development. **B.** Open siliques from the Col-2 and *ago1-Ser1001* mutant's lines. **C.** Seed weight of individual wild-type and *ago1-Ser1001* plants. *n*=3 biological replicates. Ten to fifteen plants per replicate were used for seed content measure. $p<0.05$, t-test.

To summarize, we conclude that the replacement of the highly conserved Ser1001 residue for A or D amino acids leads to the rescue of AGO1 function. However, the phospho-null mutant displays developmental phenotypes such as small rosette and delay in the juvenile to vegetative transition. On the other hand, the phospho-mimic AGO1 impacts the growth of reproductive organs and leads to a reduction in seed content in *Arabidopsis thaliana.* Our results show that the mutation of Ser1001 residue ($AGO1^{S1001A}$ and $AGO1^{S1001D}$) result in hypomorphic alleles impacting developmental process regulated by miRNAs which suggest that the integrity of the Ser1001 determines the degree of functionality of AGO1.

5.3 Part III: ARGONAUTE1 phosphorylation and the nitrogen response of *Arabidopsis thaliana*.

Since we detected AGO1 differentially phosphorylated under nitrate treatments, we asked whether Ser1001 mutation affects a whole or local- plant responses when facing a stimulus as nitrate supply or deficiency. Will it be essential for its correct growth and, therefore, for the quality of survival of plants? We have observed that AGO1 phospho-serine balance is relevant for plant development but to what extent its responses are affected by nitrogen is the question we face in this section.

5.3.1 AGO1 phospho-mutants respond differentially under nitrate treatments.

It is known that nitrate, besides its role as a nutrient, can act as a signaling molecule that regulates numerous processes including gene expression, root architecture, shoot development, seed germination, and flowering (Nacry et al., 2013; O'Brien et al., 2016; Lin and Tsay, 2017; Duermeyer et al., 2018). Within the last years, many efforts have been released to discover the molecular components of the signaling pathway triggered by nitrate. Most of the actual knowledge

has been focused on the transcriptional and post-transcriptional responses, uncovering nitrate responsive components such as receptors, several TFs, Ca^{2+}, protein kinases, among others.

The present work started with the question: how nitrate modulates early protein phosphorylation in *Arabidopsis*? On the way to answering this question, we found AGO1, one of the most important factors controlling post-transcriptional and translational gene regulation was modulated in response to nitrate. To unravel the involvement of AGO1 phosphorylation in the nitrate signaling pathway, we characterized the root response under nitrate treatments in the *ago1-1* mutant complemented with $AGO1^{S1001S}$, $AGO1^{S1001A}$, and $AGO1^{S1001D}$. Using the same experimental conditions described in the phospho-proteome profiling (Vega et al., unpublished), we evaluated the potential function of AGO1 phosphorylation in the nitrate modulation of the root system architecture. As expected, the primary root of the wild type (Col-0, Col-2, and $AGO1^{S1001S}$) was shorter when treated with KNO_3 compared to KCl 3 days after treatment. However, the primary root of the $AGO1^{S1001A}$ and $AGO1^{S1001D}$ mutants showed disturbed development (FIG 7). The *ago1-27* allele developed a longer and nitrate-insensitive primary root, while the phospho-null line was shorter and insensitive to nitrate treatments than wild-type (FIG 7 upper panel). Lateral root growth was also measured in response to the external nitrate supply. While no differences in the density of elongated lateral roots was observed, the $AGO1^{S1001A}$ mutant did not respond to nitrate, showing no effect of nitrate-induced induction of lateral roots initiation after three days treatment compared to Col-2 plants (FIG 7, middle panel). These results suggest that, under the tested experimental conditions, the phosphorylated form of AGO1 might be required for the proper primary root growth and the lateral root architecture response to nitrate in *Arabidopsis thaliana*.

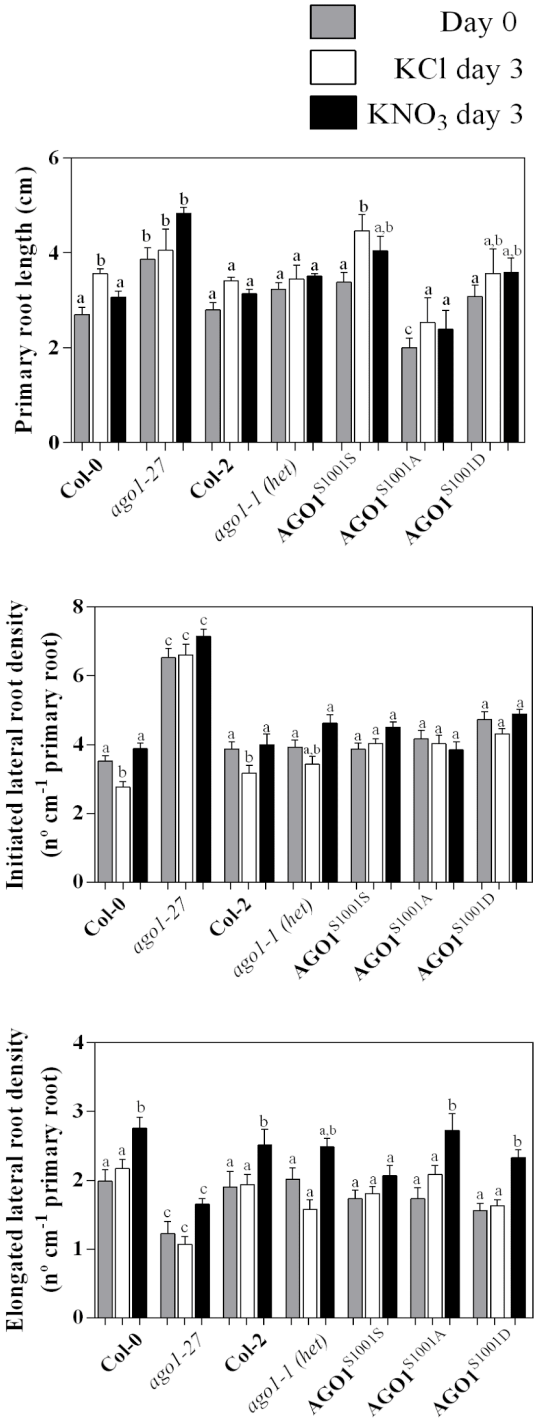


FIGURE 7. **Serine residue near PIWI domain of ARGONAUTE1 is important for the nitrate response of *Arabidopsis thaliana* roots.** Col-0, *ago1-27*, Col-2 and the AGO^{S1001S}, AGO^{S1001A} and AGO^{S1001D} plants were grown hydroponically for two weeks with ammonium as a nitrogen source. Roots were treated with 5 mM KNO_3 or 5 mM KCl as a control. After 3 days, plants were fixed in formaldehyde to measure primary root length (upper chart) and count lateral roots (LR density, middle and bottom panel). It is shown the mean and standard error for four independent replicates with at least 10 plants each. N = 4, $p < 0.05$.

The nitrate signaling pathway and the early elicited changes in Ca^{2+} and protein modification by phosphorylation have been deeply investigated in recent years (Riveras et al., 2015; Undurraga et al., 2017). Molecular factors such as kinases, TFs, and hormone signaling components are involved in controlling root development (O'Brien et al., 2016; Liu et al., 2017; Sun et al., 2017). To determine whether the altered response to nitrate in AGO1 mutants is explained by the deregulation of one or more miRNA-targets previously related to the nitrate-response of plants, we evaluated two miRNA targets which transcripts levels are regulated by nitrate in roots, the *AUXIN RESPONSIVE FACTOR 8* (*ARF8*) and *AUXIN SIGNALING F-BOX 3* (*AFB3*). The module ARF8-miR167 controls lateral root growth acting in pericycle cells. ARF8 triggers lateral root initiation and repress lateral root elongation in response to nitrogen (Gifford et al., 2008). On the other hand, miR393 and its target, AFB3, regulate primary and lateral root growth in response to nitrate (Vidal et al., 2010). Vidal et al. 2010 demonstrated that plants overexpressing miR393 or *afb3* mutant displayed reduced initiating and emerging lateral root densities compared to wild type, which showed increased lateral root growth in response to nitrate. Our results showed that $AGO1^{S1001A}$ did not respond to nitrate as wild-type and phospho-mimic mutant, showing differential levels of AFB3 but no differences in ARF8 transcript abundance (FIG 8). As expected, *AFB3* and *ARF8* mRNA levels were slightly upregulated in wild-type plants after 1-hour nitrate treatment. However, $AGO1^{S1001A}$ mutant plants showed higher levels of *AFB3* mRNA under 1-h nitrate treatments recovering to normal after 2-hours treatment (FIG 8). It is difficult to explain the obtained results since we are in the presence of a dynamic transcript behavior. However, we can suggest that altered phosphorylation of AGO1 would impact the transcriptional response of the root, particularly when faced a nitrate pulse.

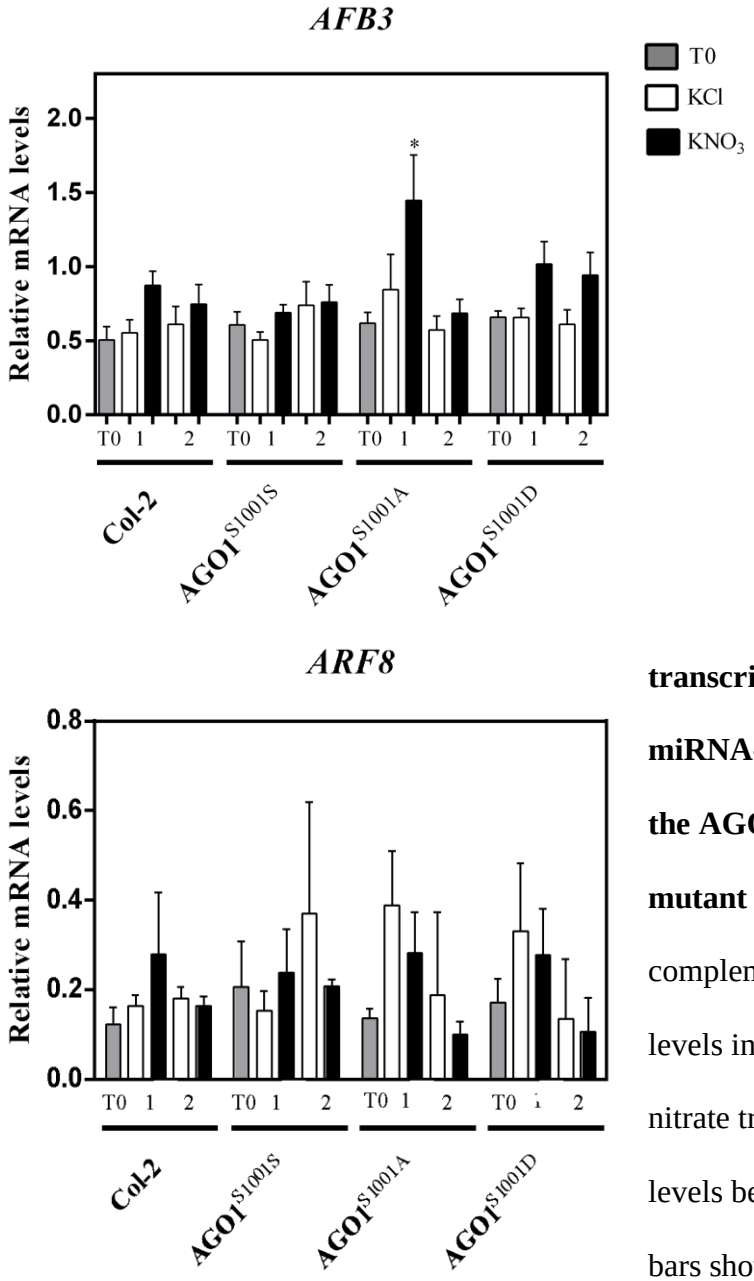


FIGURE 8. Relative transcripts levels of nitrate responsive miRNA-target genes, *AFB3* and *ARF8*, in the AGO1^{S1001S}, AGO1^{S1001A} y AGO1^{S1001D} mutant plants. Comparison between AGO1 complemented plant and Col-2 transcript levels in *Arabidopsis* roots after 1h or 2h of nitrate treatment. Grey bars indicate mRNA levels before nitrate treatments (T0), white bars show control treatment (KCl), and black bars indicate mRNA after nitrate treatments. We show four biological replicates, standard error. Asterisk show means that statistically differ ($p < 0.05$).

However, AGO1 phosphorylation would be compensated by a control system that allows the plant to modulate its molecular responses, and therefore their development efficiently and quickly.

5.3.2 AGO1 phospho-null plants have limited growth under sufficient and limiting nitrogen concentration

Since we found nitrate altered AGO1 phosphorylation pattern (FIG 1), we asked how $AGO1^{S1001A}$ or $AGO1^{S1001D}$ mutations respond to differential nitrate concentrations. To answer that question, we evaluated plant growth under limiting (0.5 mM KNO_3) or sufficient (5 mM KNO_3) nitrogen conditions. Nitrate limitation modulates root, and limits shoot growth (Nacry et al., 2013). In our work, the nitrate-limitation response was observed for wild type, $AGO1^{S001S}$, and $AGO1^{S1001D}$ mutant (FIG 9). Interestingly, $AGO1^{S1001A}$ arrested growth regardless of nitrate availability. As it was observed for standard growth conditions, $AGO1^{S1001A}$ developed a reduced shoot area showing no differences according to time or nitrate availability in the medium (FIG 3A and 9A-B).

Normally, under mild nitrogen limitation, primary and lateral root length is increased in wild type plants (Giehl et al., 2014; Sun et al., 2017). However, in our analysis, $AGO1^{S1001A}$ and $AGO1^{S1001D}$ mutants did not show longer roots when they were grown in a vertical plate under 5 mM KNO_3 as compared to wild-type plants (FIG 9C-D). Also, we observed that both limiting and sufficient N concentrations stimulate primary root growth of $AGO1^{S1001D}$ plants (FIG 9C-D). These results show that altered AGO1 phosphorylation impaired shoot and root architecture under sufficient N, suggesting that AGO1 phosphorylation might control developmental responses of plants to environmental cues such as nitrate availability in the soil.

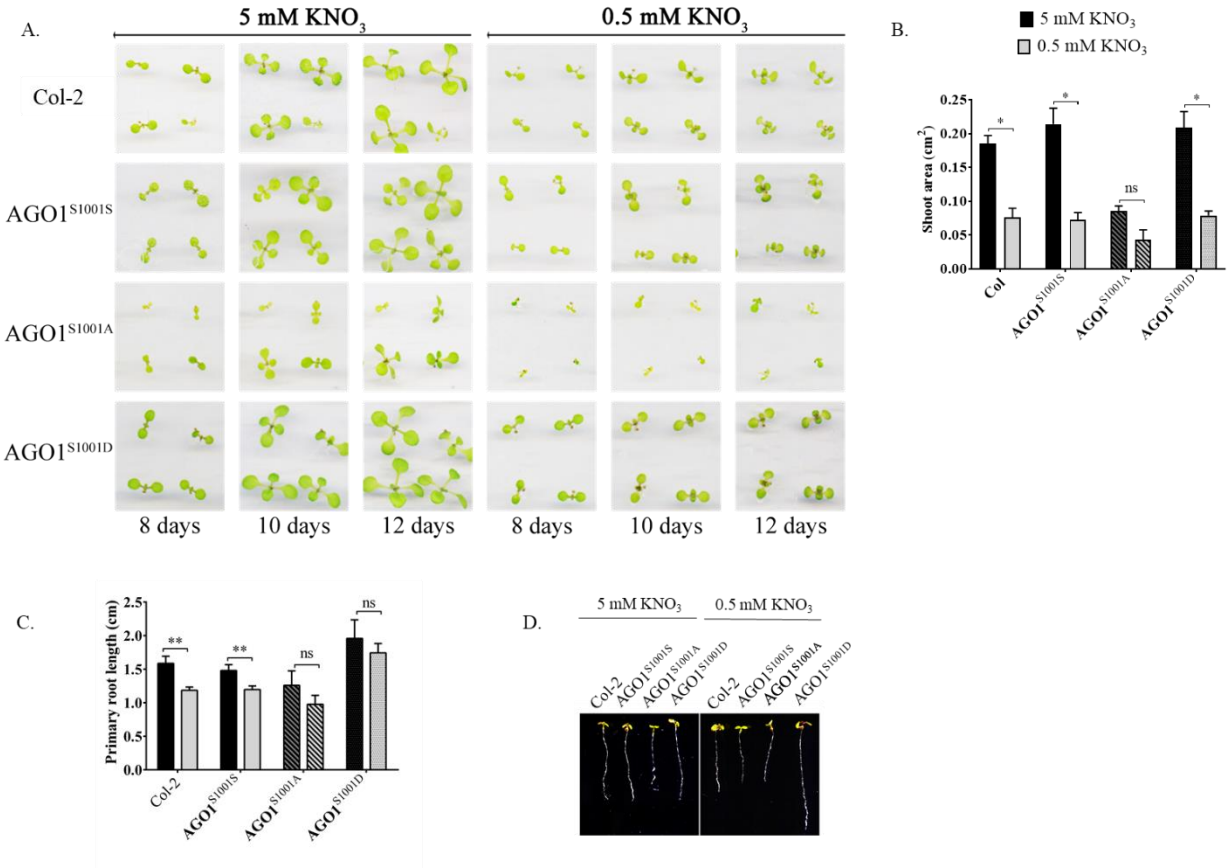


FIGURE 9. Alteration in AGO1 phosphorylation impaired shoot and root development under sufficient N concentration. A. A representative set of 8, 10 and 12 days-old plants grown in free-N MS medium supplemented with 0.1 % sucrose and 5 (left panel) or 0,5 mM KNO_3 (right panel) as a nitrogen source is shown. **B.** Plot showing the shoot area of 12 days-old wild type and phosphor defective mutant plants. **C-D**. The primary root length of 7 days-old plants was measured. Wild-type (Col and $AGO1^{S1001S}$), phospho-defective ($AGO1^{S1001A}$) and phospho-mimic ($AGO1^{S1001D}$) genotypes plants were used. Three biological replicates were analyzed, > 60 plants were measured for each replicate. Asterisk indicates the significant differences among the genotypes using two-way ANOVA, $p < 0.0001$. ns means no significative differences between genotypes.

5.4 PART IV: The mechanistic function behind AGO1 phosphorylation.

We showed that AGO1 phospho-null mutant plants are smaller and non-responsive to nitrate. On the other hand, AGO^{S1001D} growth is practically normal until the end of the lifecycle, showing abnormal siliques and lower seed production than wild type. To get insights about the role of AGO1 phosphorylation, the next step was to investigate AGO1 molecular function. To evaluate whether miRNA biogenesis, miRNA:AGO1 association, or miRNA target levels were altered by mutations in AGO1 Ser1001, small RNA, AGO1-bounded sRNA, and total RNA libraries from *Arabidopsis* wild-type (Col-2 and AGO1^{S1001S}) and transgenic plants expressing either AGO1^{S1001A} or AGO1^{S1001D} were analyzed. Additionally, we evaluated the slicer activity of AGO1 *in vitro*.

The results showed here allows us to conclude that miRNA biogenesis and loading are not altered by changes in AGO1 Ser1001 phosphorylation. However, the importance of the Ser1001 residue lies in the RISC function, affecting the transcriptome response and the slicer activity associated with the phosphorylated status of AGO1.

5.4.1 AGO1 phosphorylation does not influence accumulation of mature miRNAs.

AGO1 protein plays a role not only in processing mRNA molecules but also influences transcription, stabilization, and degradation of miRNAs (Vaucheret et al., 2004; Vaucheret et al., 2006; Diederichs and Haber, 2007; Dolata et al., 2016). miRNA levels are reduced in strong *ago1* alleles, indicating that AGO1 is necessary to maintain the integrity of the miRNA accumulation (Vaucheret et al., 2004; Arribas-Hernández et al., 2016a). However, hypomorphic *ago1-27, ago1-26, and ago1-25* alleles show normal accumulation and RISC-binding of miRNAs (Vaucheret et al., 2004; Carbonell et al., 2012). To evaluate whether AGO1 Ser1001 residue has a role in miRNA

abundance, deep sequencing of total sRNA was performed for AGO1 phospho-defective, phospho-mimic, and wild type plants. In addition, we corroborated the accumulation of highly expressed miRNAs in these genotypes in both root and shoot organs by northern blots.

Sequencing using Illumina technology yielded 14 to 19 million reads from *Arabidopsis* AGO1^{S1001A}, AGO1^{S1001D} or wild type (Col-2 and AGO1^{S1001S}) plants. Around 70% of total reads remained after removal of adaptor sequences (10–13 million per sample) and were processed further. Approximately 78–92% of the reads were mapped perfectly to the *Arabidopsis* genome and included in our analysis. Analysis of total sRNA detected 335 known miRNAs, which represents 78,45% of the total 427 miRNAs registered in miRbase (without any threshold of the read number per miRNA). Known miRNAs with more than twenty reads considering the three biological replicates, represent almost 56 % of the miRNA database. As reported previously, total sRNAs sequenced were dominated by 21-nt and 24-nt long species (24 – 28%), with the population of 24-nt sRNAs much larger than the 21-nt ones (FIG 10A).

Our analysis showed that in total sRNA fraction miRNAs remained unchanged in AGO1^{S1001A} and AGO1^{S1001D} mutants relative to wild-type (Col-2 and AGO1^{S1001S}), suggesting that the mutation in the Ser1001 residue of AGO1 does not affect miRNA biogenesis or accumulation (FIG 10B). These results were confirmed by checking some highly expressed miRNA (miR156, miR172, and miR390, and miR393) by northern blot analysis (FIG 11). Consistent with what was detected by sRNA deep sequencing, these key miRNAs involved in developmental processes were unaffected by changing the AGO1 Ser1001 residue.

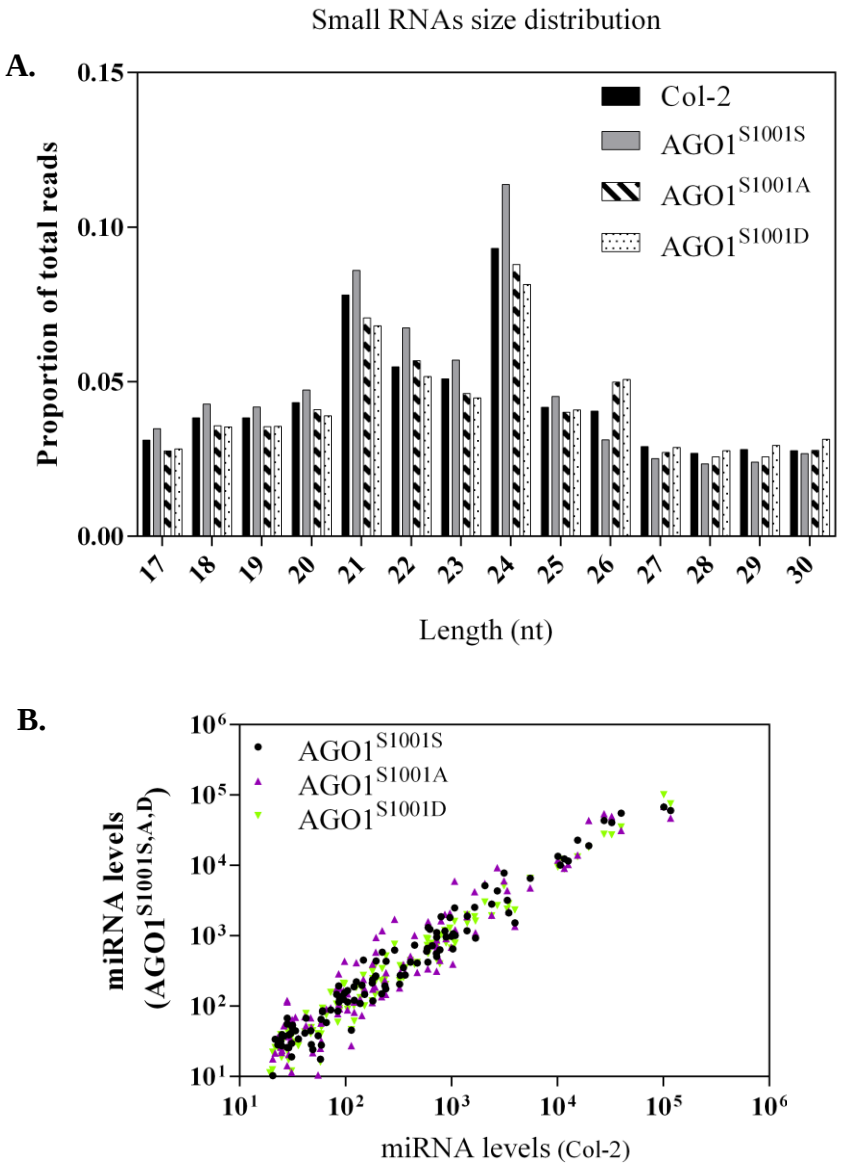


FIGURE 10. Profiling of miRNA population of phospho-defective and phospho-mimic plants. A. Reads-size distribution of the sequenced small RNA from Col, AGO1^{S1001S}, AGO1^{S1001A}, and AGO1^{S1001D} plants. **B.** Scatter plot showing the correlation in the abundance of total small RNAs between wild-type and AGO1 phospho-null plants. Small RNA seq was made in three biological replicates, libraries were constructed using NEB next multiplex small RNA library Prep set for Illumina. Each dot represents a unique miRNA.

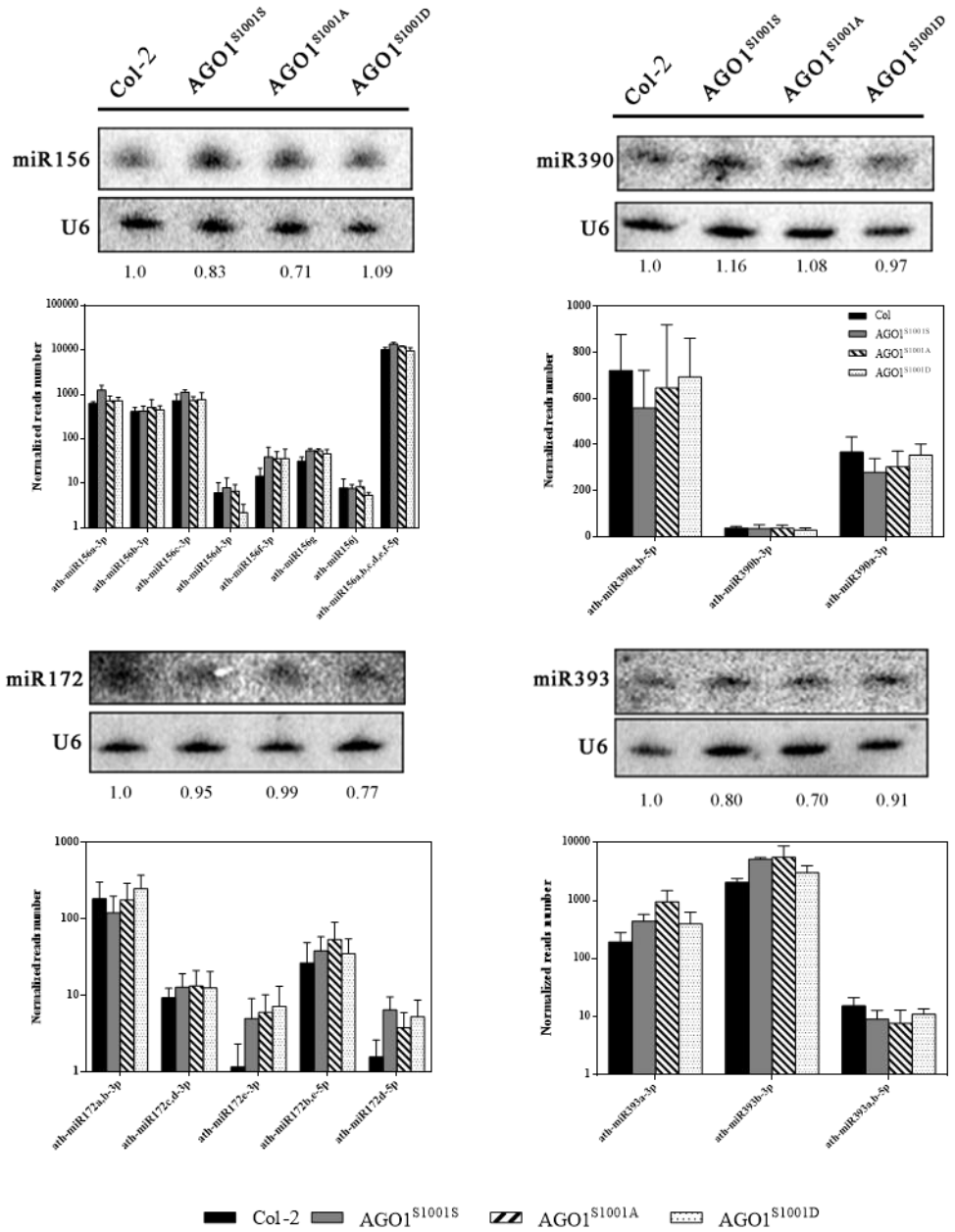


FIGURE 11. Phosphorylation of AGO1 does not alter the abundance of miRNAs involved in the development and response to nitrate of *Arabidopsis*. miR156, miR172, miR390 and miR393 accumulation in wild type (Col-2, AGO1^{S1001S}), AGO1^{S1001A}, and AGO1^{S1001D} *Arabidopsis* seedlings. For each miRNA, specific oligonucleotides probes were used in small RNA gel blot analysis starting from total RNA from Ser1001 mutants and wild type seedlings. U6 small nuclear ribonucleoprotein was used as a loading control. Bottom plots indicate different miRNA species of the same miRNA detected by deep sequencing. The mean and standard error for three biological replicates is shown.

5.4.2 Serine1001 alteration does not affect miRNA binding to AGO1.

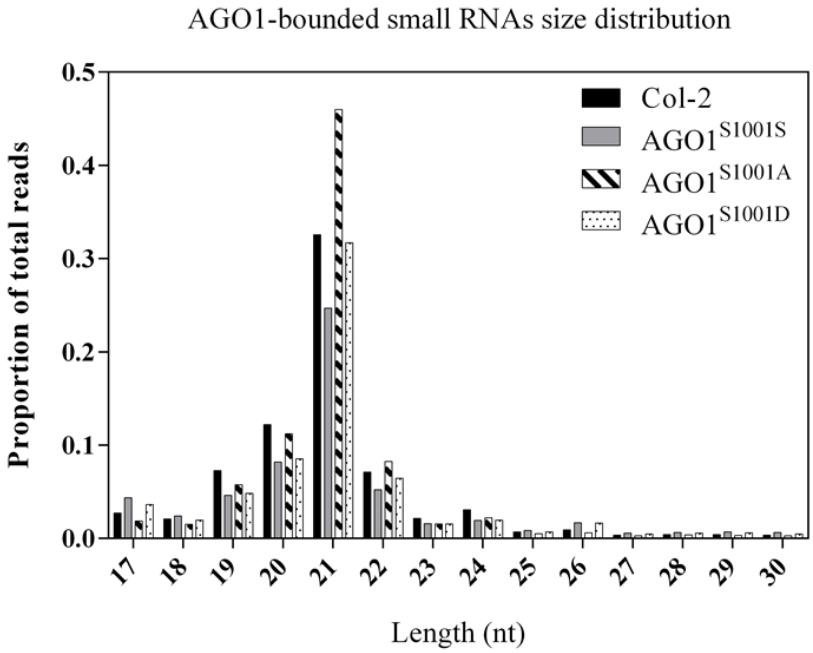
Loading of miRNAs into AGO1 protein is key during the formation of a functional RISC complex. While total miRNA accumulation was not affected in AGO1^{S1001S}, AGO1^{S1001A}, and AGO1^{S1001D} mutants, we wondered whether miRNA loading into AGO1 could be impaired or altered due to AGO1 phosphorylation status. To assess this question, we investigated the role of the Ser1001 residue on miRNA loading onto AGO1 by sequencing small RNAs from purified AGO1 complexes of Col-2 and *ago1-1* complemented AGO1^{S1001S}, AGO1^{S1001A}, and AGO1^{S1001D} seedlings. Each sample yielded approximately 17 million total reads before trimming. After trimming, around 70% of reads remained with lengths between 15 and 40-nt. Seventy-two to ninety-five percent of the reads were mapped perfectly to the *Arabidopsis* genome and were included in our analysis. Our results revealed that between 25 to 46% of the AGO1-associated sRNAs species were 21-nt in length, and only 2-3% were 24-nt (FIG 12) which is consistent with what has been previously described for plant AGO1-associated miRNAs (Mi et al., 2008; Liu et al., 2018).

Within the total miRNAs known in the miRBase, 55.3% (236 out of 427 miRNAs) were detected in our analysis. miRNAs species associated with AGO1 phospho-mutants as in wild-type plants grown under complete nutrient conditions (FIG 12). While altering Ser1001 residue has an impact on *Arabidopsis* growth, our results indicate that AGO1 phosphorylation does not alter total miRNA loading onto AGO1 under our experimental conditions. These results are consistent with previous studies obtained for point mutation in other residues close to the serine studied here (Carbonell et al., 2012).

These results suggest that the pathway for miRNA biogenesis, accumulation and loading might be intact in AGO1 phospho-mutants. The results shown here are consistent with other reports in which the effect of mutations close to the PIWI domain has been studied.

Despite miRNA association to AGO1 cannot explain the vast phenotypes we observed in this work. The presented results show that, although the mutation of the phospho-site of AGO1 does not alter miRNA accumulation, it seems that it could impact the way plants respond to an external stimulus such as nitrate.

A.



B.

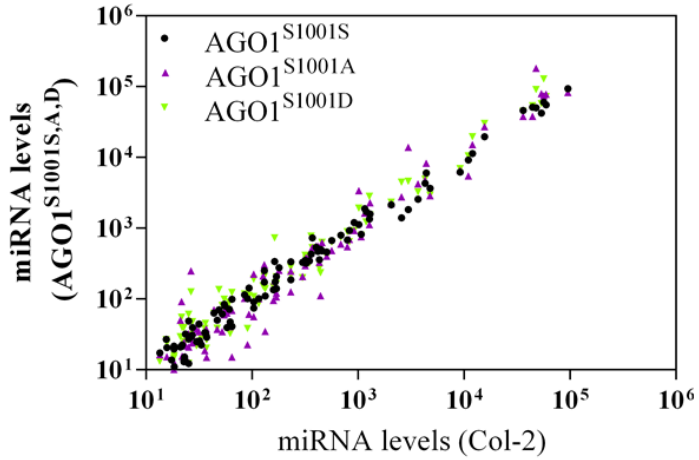


FIGURE 12. Profiling of miRNA population bounded to phospho-defective and phospho-mimic plants. A. Reads-size distribution of the sequenced bounded-sRNAs to wild-type Col-2 and the *ago1-1* complemented AGO1^{S1001S}, AGO1^{S1001A}, and AGO1^{S1001D} complexes. **B.** Scatter plot showing the correlation in the abundance of AGO1-associated small RNAs between wild-type and AGO1 phospho-null plants. Small RNA seq was made in three biological replicates, libraries were constructed using NEB next multiplex small RNA library Prep set for Illumina. Each dot represents a unique miRNA.

5.4.3 AGO1 phosphorylation deficiency alter gene transcript accumulation pattern in *Arabidopsis.*

We have previously described the phenotypic and molecular characteristics of AGO1 phospho-null and phospho-mimic plants. We observed $AGO1^{S1001A}$ plants have developmental defects and respond differentially to variation in N supply. However, miRNA abundance and AGO1-loading are not impaired, other mechanisms might explain such phenotypes.

Regarding the function of the AGO1 phosphorylation site Ser1001 here we address two important biological questions: 1. To what extent mRNA levels change in the mutants as compared to wild type plants? And 2. Within the observed mRNA level changes, is there a relationship between miRNA-targets relative abundance and the phenotypic responses observed of AGO1 phospho-mutants?

To understand the impact and function of AGO1 phosphorylation, we measured mRNA levels in the different genotypes (Col-2, $AGO1^{S1001S}$, $AGO1^{S1001A}$, and $AGO1^{S1001D}$) and constructed a regulatory network encompassing miRNA-mediated post-transcriptional mechanism of control of gene expression during plant responses to N availability. First, we sequenced the transcriptome of 12-days old AGO1 phospho-mutants ($AGO1^{S1001A}$ and $AGO1^{S1001D}$) and wild type (Col-2 and $AGO1^{S1001S}$) seedlings grown under full nutrient (MS 50%), sufficient (5 mM KNO_3) and limiting (0.5 mM KNO_3) nitrate concentrations.

To identify differentially expressed genes, we used the median normalization method followed by a two-way analysis of variance (ANOVA) to determine if an interaction between genotype and treatment exists (described in Materials and methods). We obtained a total of 490 genes whose expression changes regarding Col-2 in any of the AGO1 mutants analyzed or in each nitrate condition used (FIG 13). From the list obtained, we found most genes were regulated by

the growth condition (C) in the phospho-null mutant (FIG 13A). That means, from the 490 DE genes, 444 showed differential mRNA levels in response to nitrate concentration (C), while 305 were differentially accumulated only because of the phospho-site variable (G). The most interesting list of genes arises from the C:G list, in which transcript accumulation depends on genotype and nitrogen condition. For instance, when one gene is up-regulated under limiting nitrate condition, it is repressed when the plant is grown under sufficient nitrate concentration. By the previous analysis, treatment was found as the main variation source between samples.

The $AGO1^{S1001A}$ mutant showed to be the most responsive genotype. Seventy five percent of genes were deregulated in response to limiting nitrate (0.5 mM KNO_3) when compared with the control growth medium (full nutrient, MS 50% = 0.95 mg/L KNO_3, 0.825 mg/L NH_4NO_3) (FIG 13C). Following the same behavior, when we analyzed the number of genes regulated in each genotype, we observed that most of the responsive genes were from the phospho-null mutant (FIG 13B), which is consistent with the degree of phenotypic defects previously observed. On the other hand, we found a similar number of responsive genes regulated by the availability of nitrate in the $AGO1^{S1001S}$ and $AGO1^{S1001D}$ mutant. However, very few genes were regulated in the $AGO1^{S1001D}$ when comparing with wild type (FIG 13B, C).

According to nitrate availability, almost half (49%) of the genes listed as G:C responsive are deregulated by the contrasting nitrate concentration (5/0.5 mM), which means that nitrate responsive genes showed altered mRNA levels in the AGO1 Ser1001 mutant alleles (FIG 13D). From that number, contrasting nitrate concentration lead a hundred transcripts differentially accumulated in the AGOS1001A mutant, relative to the $AGO1^{S1001D}$. Interestingly, twenty-seven of those genes have been described to be targeted by one or more miRNA (FIG 13D).

When looked at the list of responsive genes in the mutants, 244 were found to be deregulated in the AGO1^{S1001A} mutant compared to wild type (FIG 13). From that number, 166 (68%) showed higher transcript levels than wild-type, and only 78 (32%) were repressed. On the contrary, 20 (59%) out of 34 genes were down-regulated in the AGO1^{S1001D} mutant (FIG 13 and FIG 14). These results indicate that mRNA levels are more sensitive to nitrate deficiency on plants expressing AGO1^{S1001A}.

Looking deeper, 56% of the genes responding to either nitrate condition or Ser1001 mutation, were up-regulated (FIG 14). If we compare mRNA level of plants growing in 5 mM KNO_3 and full nutrient condition, we can observe that only sixteen and fifteen genes are up or down regulated in the mutants, respectively (FIG 14, left panel). However, 0.5 mM nitrate conditions show a different scenario. In this context, 59 and 134 genes show higher and lower mRNA abundance in the AGO1^{S1001A} mutant, comparing to wild type. Moreover, similar behavior is observed in the AGO^{S1001A} mutant relative to the AGO1^{S1001D} (FIG 14, right panel). Dissecting the list of the 127 responsive genes, 106 were down-regulated in the phospho-null mutant comparing either to wild-type or the AGO1^{S1001D} mutant. Interestingly, only one gene showed to be down-regulated in the AGO1^{S1001D} mutant under this experimental condition. The previous analysis suggests that most of the nitrate responsive genes are down-regulated in the phospho-null mutant, which could mean that the phosphorylation of AGO1 is a necessary step to ensure the full and correct response to low nitrate availability.

One interesting observation is that under contrasting nitrate conditions (5/0.5 mM KNO_3), the AGO1^{S1001A} mutant displayed the highest responsive gene number, showing 65 and 140 transcripts up and down-regulated, respectively (FIG 13E and FIG 14B-C). These results showed that nitrate regulates AGO1 phosphorylation, and such regulation is significative altered when the

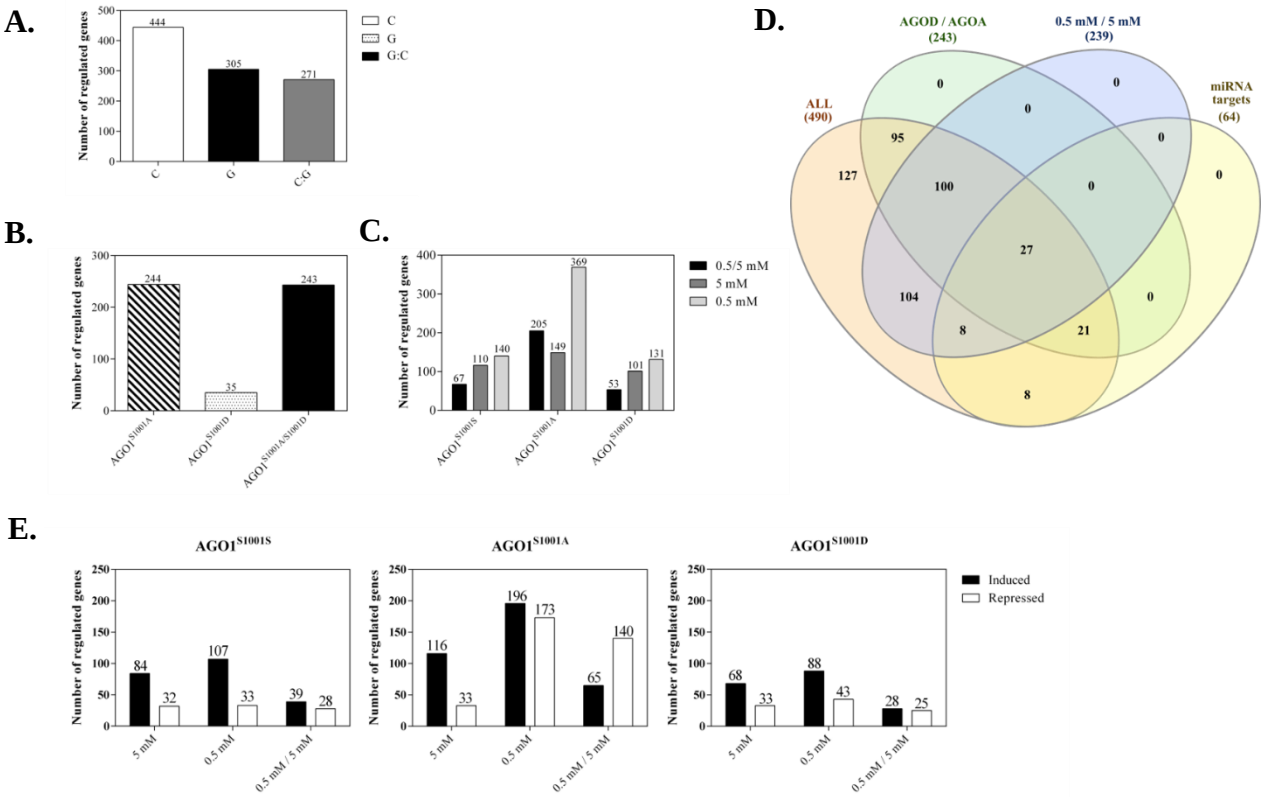


FIGURE 13. The nitrate concentration is the main source of variation in the mRNA levels response of AGO1 mutants. **A.** The number of genes regulated by the growth condition (C), genotype (G) and those regulated by the intersection condition: genotype (G:C) **B.** Dissection of the number of differentially accumulated gene transcripts in each genotype ($AGO1^{S1001S}$ was used as a reference), and growth condition (**C**). **D**. Venn diagram representing intersection between differentially accumulated transcripts in AGO1 mutants and targets of miRNAs. **E.** Number of genes up and down regulated in each genotype growth under sufficient and limiting nitrate concentration. Differentially expressed genes were normalized using Col-2 as control. Two-way ANOVA was used for statistical analysis using $p < 0.01$. In the 0.5/5 mM, we compared mRNA levels in 0.5 mM KNO3 relative to 5 mM, then, we compared mRNA abundance under 5 and 0.5mM KNO_3 relative to full nutrient (MS 50%) condition.

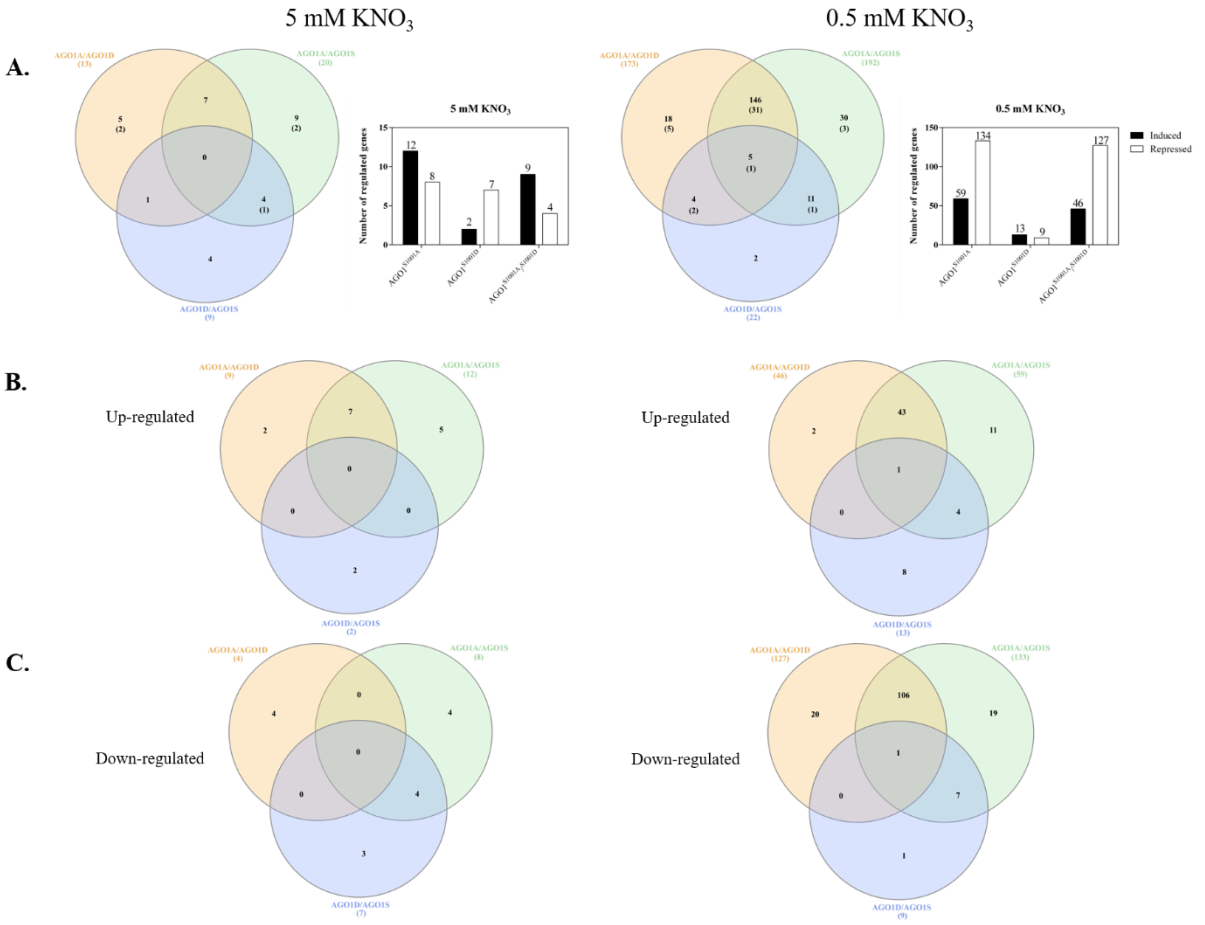


FIGURE 14. Effects of AGO1^{S1001A} and AGO1^{S1001D} mutations on the accumulation of transcripts under contrasting nitrate concentration. **A.** Left: Overlap of transcripts regulated between AGO1^{S1001A}, AGO1^{S1001D} and AGO1^{S1001S}. Numbers in brackets indicate the numbers of predicted miRNA targets. Right: Number of regulated genes between wild type (Col-2) and phospho-mutants. The number of genes shared between lists is shown inside the ovals. **B & C.** Overlap between transcripts up- and down-regulated in AGO1 phospho-mutants under contrasting nitrate concentrations (5 mM and 0.5 mM KNO_3), respectively.

Ser1001 residue is replaced by an alanine. Within this context, we positioned the AGO1^{S1001A} as the most responsive mutant when the plant face contrasting nitrate concentrations in the medium.

5.4.4 miRNA-target genes coding for nucleic acid binding proteins, catalytic activity, and transcription factors result in abnormal mRNA levels in AGO1 phospho-mutants.

Intending to uncover the impact of AGO1 phosphorylation pattern on the transcript abundance of miRNA targets, we asked how many of the regulated genes (C:G) are miRNA targets. From the list of genes whose mRNA level is regulated by the interaction of nitrogen condition and genotype (G:C), 64 genes were found to be targeted by at least one miRNA (FIG 14D and TABLE N° 3). To evaluate the biological significance of the miRNA-target deregulated genes in the AGO1 mutants, we analyzed overrepresented biological terms in the group of genes whose transcript pattern depends on the nitrogen condition and the phosphorylation status of AGO1. Despite no GO terms overrepresentation was obtained, we observed that 32% correspond to genes involved in either "biological regulation" (16%) or "metabolic process" (16%) categories (FIG 15A, C). Interestingly, from twenty miRNA-target genes, four of them codify for nucleic acid binding proteins, one corresponds to a calcium-binding protein, and 15% codify for TFs (FIG 15B). Looking deeper into this list, we found "transporters", "hydrolase", "oxidoreductase" and "transferase" as responsive elements to our conditions. It is worth mentioning that genes listed in TABLE N°3 were selected because their expression is not only modulated by miRNAs, but also dependent on AGO1 phosphorylation status. For example, genes that codify for proteins involved in plant development (*SEPALLATA 3*), sugar metabolism (*BFRUCT4*), and response to stress (*OXS3*), behave oppositely among AGO1 mutants (FIG 16). Consistent with our previous results, the AGO1^{S1001D} mutant behaves like wild-type, showing not great changes in the gene transcript

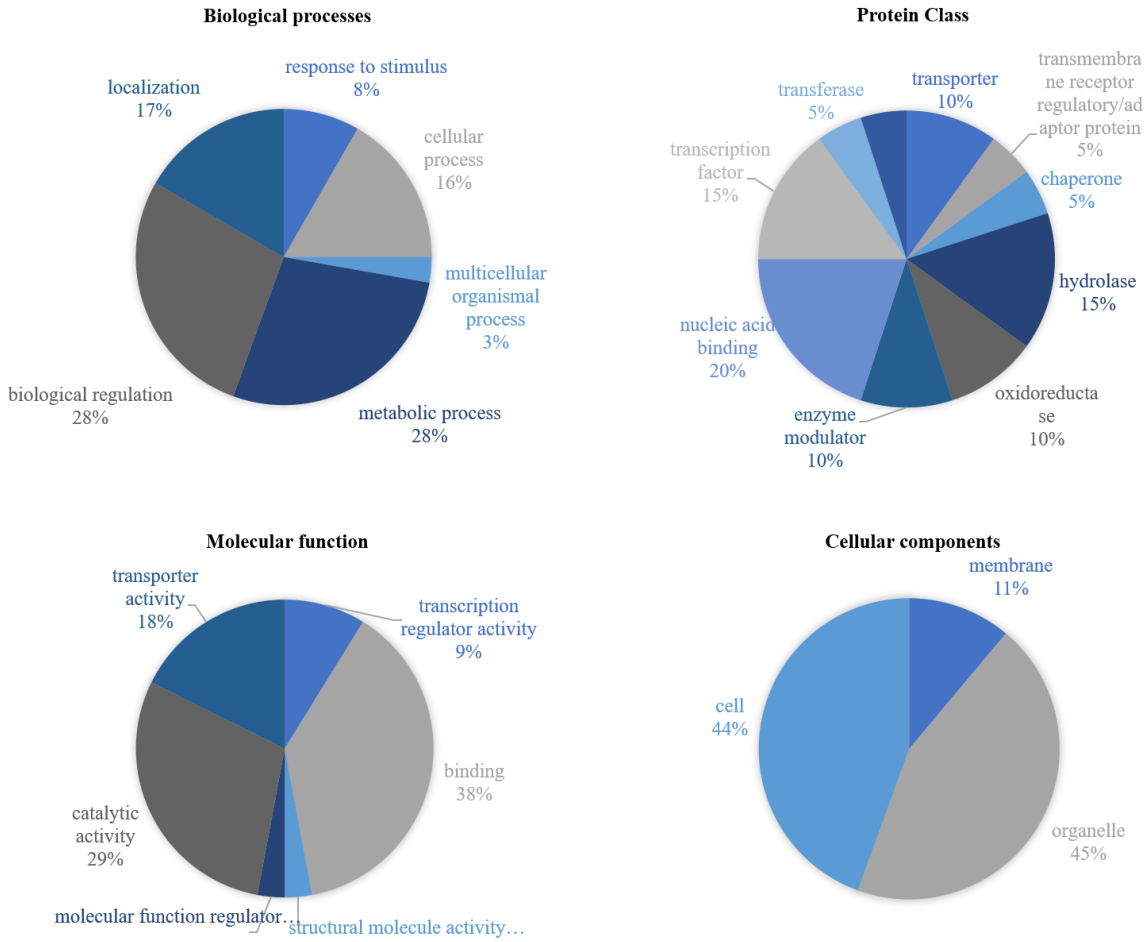


FIGURE 15. Gene ontology enrichment analysis of genes regulated by contrasting nitrate concentration (C) and AGO1 phospho-genotype (G) (C:G). The differentially expressed genes were analyzed by enrichment analysis using PANTHER Classification System. GO categories in biological process, protein class, molecular function, and cellular component are shown in yellow and orange (false discovery rate, FDR < 0.05).

TABLE N° 3. miRNA-target genes regulated by nitrate availability and AGO1 phosphorylation status.

Gen	Symbol	Description	miRNA	Regulated in
AT1G08035	AT1G08035	hypothetical protein	ath-miR870	
AT1G10120	AT1G10120	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	ath-miR396b	D/A0.5
AT1G12240	ATBETAFRUCT4	Glycosyl hydrolases family 32 protein	ath-miRf10765	D/AMS
AT1G15520	ABCG40	pleiotropic drug resistance 12	ath-miRf10376	5/0.5, D/A0.5
AT1G16310	AT1G16310	Cation efflux family protein	ath-miRf10561	5/0.5, D/A0.5
AT1G20700	WOX14	WUSCHEL related homeobox 14	ath-miR1888	D/A0.5
AT1G24260	SEPT3	K-box region and MADS-box transcription factor family protein	ath-miRf10694	D/AMS
AT1G26380	AT1G26380	FAD-binding Berberine family protein	ath-miRf10630	5/0.5
AT1G28470	NAC010	NAC domain containing protein 10	ath-miRf10115	5/0.5, D/A0.5
AT1G32960	SBT3.3	Subtilase family protein	ath-miRf11045	5/0.5, D/A0.5
AT1G33960	AIG1	P-loop containing nucleoside triphosphate hydrolases superfamily protein	ath-miRf10320	5/0.5, D/A0.5
AT1G48040	AT1G48040	Protein phosphatase 2C family protein	ath-miR856|ath-miRf10682	5/0.5
AT1G53625	AT1G53625	hypothetical protein	ath-miR414	5/0.5, D/A0.5
AT1G61080	AT1G61080	Hydroxyproline-rich glycoprotein family protein	ath-miRf10701	5/0.5, D/A5
AT1G66690	AT1G66690	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	ath-miR163	
AT1G67900	AT1G67900	Phototropic-responsive NPH3 family protein	ath-miRf10310-|ath-miRf10645	D/AMS
AT1G68440	AT1G68440	transmembrane protein	ath-miR414	5/0.5, D/AMS
AT1G68670	AT1G68670	myb-like transcription factor family protein	ath-miR838|ath-miRf10115	
AT1G69290	AT1G69290	Pentatricopeptide repeat (PPR) superfamily protein	ath-miRf10993	
AT2G31230	ERF15	ethylene-responsive element binding factor 15	ath-miRf10440	D/A0.5
AT2G31940	AT2G31940	oxidoreductase/transition metal ion-binding protein	ath-miRf10427	5/0.5, D/A0.5
AT2G38860	YLS5	Class I glutamine amidotransferase-like superfamily protein	ath-miR414	5/0.5, D/A0.5
AT2G44940	AT2G44940	Ethylene-responsive transcription factor 34	ath-miRf10896	5/0.5, D/A0.5

AT2G45550	CYP76C4	cytochrome P450, family 76, subfamily C, polypeptide 4	ath-miRf10997	5/0.5, D/A0.5
AT3G05690	NF-YA2	nuclear factor Y, subunit A2	ath-miR169d,e,f,g,h,I,j,k,l,m,n	D/A0.5
AT3G06370	NHX4	sodium hydrogen exchanger 4	ath-miR398a	5/0.5, D/A0.5, D/AMS
AT3G09220	LAC7	laccase 7	ath-miR857	5/0.5, D/A0.5
AT3G12750	ZIP1	zinc transporter 1 precursor	ath-miRf10106-|ath-miRf10748	
AT3G16890	PPR40	pentatricopeptide (PPR) domain protein 40	ath-miR413	5/0.5
AT3G21755	AT3G21755	Natural antisense transcript overlaps with AT3G21760	ath-miR414	
AT3G24310	MYB305	myb domain protein 305	ath-miR858	D/A0.5
AT3G28570	AT3G28570	P-loop containing nucleoside triphosphate hydrolases superfamily protein	ath-miRf10357	5/0.5
AT3G30210	MYB121	myb domain protein 121	ath-miR858	D/A0.5
AT3G42180	AT3G42180	Exostosin family protein	ath-miRf11134	5/0.5
AT3G49780	PSK4	phytosulfokine 4 precursor	ath-miRf10008	
AT3G55370	OBP3	OBF-binding protein 3	ath-miRf10440-|ath-miRf10704	5/0.5, D/A0.5
AT3G62460	AT3G62460	Putative endonuclease or glycosyl hydrolase	ath-miRf11151	D/A0.5
AT4G00870	AT4G00870	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	ath-miRf11030	5/0.5, D/A0.5
AT4G01010	CNGC13	cyclic nucleotide-gated channel 13	ath-miRf10448	D/A0.5
AT4G04990	AT4G04990	serine/arginine repetitive matrix-like protein (DUF761)	ath-miRf11092	5/0.5, D/A0.5
AT4G10890	AT4G10890	DDE family endonuclease	ath-miRf10447|ath-miRf10931	
AT4G18160	KCO6	Ca2 activated outward rectifying K channel 6	ath-miRf10533	
AT4G33330	PGSIP3	plant glycogenin-like starch initiation protein 3	ath-miR414|ath-miRf11195	
AT4G34590	GBF6/bZIP11	G-box binding factor 6	ath-miR1886	5/0.5
AT4G34860	A/N-InvB	Plant neutral invertase family protein	ath-miRf10015|ath-miRf10087-|ath-miRf10545|ath-miRf10896	5/0.5, D/A0.5
AT4G38480	AT4G38480	Transducin/WD40 repeat-like superfamily protein	ath-miR414|ath-miRf10369	
AT5G02490	Hsp70-2	Heat shock protein 70 (Hsp 70) family protein	ath-miR855	5/0.5, D/A0.5
AT5G15760	PSRP3/2	Ribosomal protein PSRP-3/Ycf65	ath-miRf10764	
AT5G19470	NUDT24	nudix hydrolase homolog 24	ath-miRf10860|ath-miRf11150	D/A0.5
AT5G19880	AT5G19880	Peroxidase superfamily protein	ath-miRf10313	5/0.5
AT5G22090	AT5G22090	FAF-like protein (DUF3049)	ath-miRf11101	5/0.5
AT5G24200	AT5G24200	alpha/beta-Hydrolases superfamily protein	ath-miRf10255|ath-miRf10764	5/0.5, D/A0.5
AT5G39850	AT5G39850	Ribosomal protein S4	ath-miR414	D/A0.5

AT5G42500	AT5G42500	Disease resistance-responsive (dirigent-like protein) family protein	ath-miRf10088|ath-miRf10219-|ath-miRf10352|ath-miRf10457|ath-miRf10458|ath-miRf10733|ath-miRf10804|ath-miRf11079	5/0.5, D/A0.5
AT5G43360	PHT1;3	phosphate transporter 1;3	ath-miRf10453	5/0.5, D/A5
AT5G44460	CML43	calmodulin like 43	ath-miRf10920	
AT5G49800	AT5G49800	Polyketide cyclase/dehydrase and lipid transport superfamily protein	ath-miRf10429	
AT5G49870	AT5G49870	Mannose-binding lectin superfamily protein	ath-miR846	
AT5G53660	GRF7	growth-regulating factor 7	ath-miR396a|ath-miR396b|ath-miR447c	D/A0.5
AT5G56550	OXS3	oxidative stress 3	ath-miR414	D/AMS
AT5G58780	cPT5	Undecaprenyl pyrophosphate synthetase family protein	ath-miRf10751	5/0.5, D/A0.5
AT5G61520	AT5G61520	Major facilitator superfamily protein	ath-miRf10686	D/A0.5
AT5G61790	CNX1	calnexin 1	ath-miRf10115-|ath-miRf10646	5/0.5, D/A0.5
AT5G63225	AT5G63225	Carbohydrate-binding X8 domain superfamily protein	ath-miRf10993	5/0.5, D/A0.5

ath = *Arabidopsis thaliana*, 5 = 5 mM KNO_3, 0.5 = 0.5 mM KNO_3, D = $AGO1^{S1001D}$, A = $AGO1^{S1001A}$

List of genes that are target of miRNAs and which mRNA accumulation pattern is altered by the interaction between the growth condition and the AGO phosphorylation site mutation (C:G). Column "**Regulated in**" shows comparison between nitrate concentration (5 or 0.5 mM KNO_3) and AGO1 phospho-mutant (A or D) relative to wild-type. For example, the LAC7 gene is deregulated under limiting nitrate (0.5 mM KNO_3) in the $AGO1^{S1001A}$ mutant when comparing with the $AGO1^{S1001D}$ mutant. This gene also responds to contrasting nitrate concentration (5/0.5).

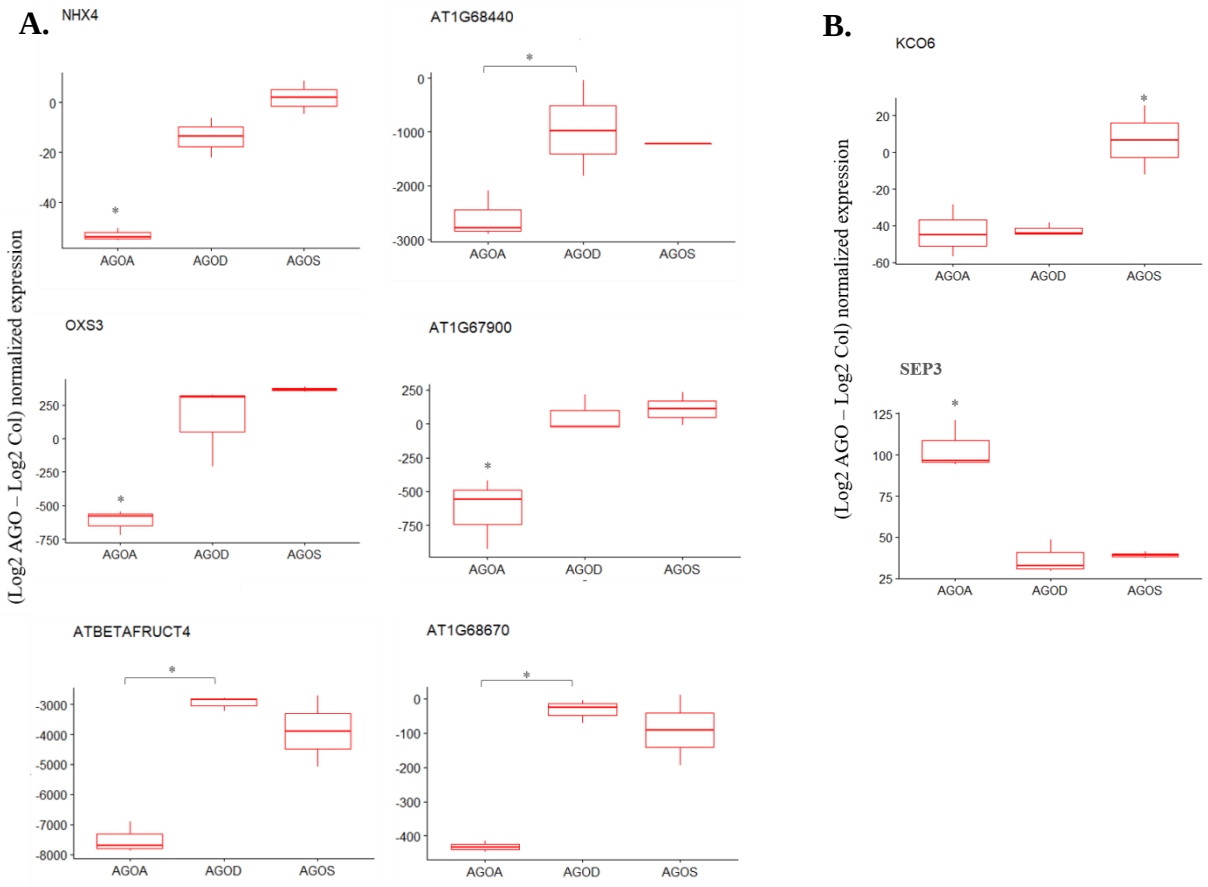


FIGURE 16. Normalized mRNA levels of genes that respond differentially to AGO1 phosphorylation growing under full nutrient condition. The box plot shows Col-2 normalized reads subtraction to every AGO1 genotype. A number >0 indicates that the mutant (AGO1) showed more reads than Col-2. **A.** Down-regulated genes in the AGO1^{S1001A} mutant relative to AGO1^{S1001D} and AGO1^{S1001S} (wild-type). **B.** Shows mRNA accumulation of genes coding for *KCO6* (upper panel) and *SEP3* (bottom panel). *KCO6* is down-regulated in both AGO1 mutant while *SEP3* in up-regulated under full-nutrient conditions.

levels, opposite to what was observed for the phospho-null mutant (FIG 16).

These results correlate with the developmental phenotypes previously described for the phospho-null mutants. Moreover, the number of genes whose mRNA levels are altered can be used as an indicator of AGO1 activity under our experimental conditions.

The previous analysis allowed us to identify miRNA-target genes whose transcripts are differentially accumulated in the phospho-mutants and have a role during plant development and response to environmental stimuli. Finally, we showed that AGO1 Ser1001 residue might alter the expression of genes that code for a diverse class of proteins involved in important biological processes with molecular functions such as transport, transcriptional regulation, and nucleotide-binding (FIG 15 and FIG 16).

5.4.5 AGO1 phosphorylation alters miRNA-target mRNA accumulation depending on nitrate concentration.

In the previous analysis, we selected those genes whose transcript levels were differentially altered by nitrate availability in the AGO1 mutants relative to wild-type plants (Col-2). To understand how nitrate concentration is altering transcript abundance of miRNA targets, genes whose transcripts accumulate differentially in the $AGO1^{S1001A}$ or $AGO1^{S1001D}$ genotype compared to $AGO1^{S1001S}$ genotype were selected. We obtained a list of 36 genes showing altered transcript accumulation in response to nitrate in the $AGO1^{S1001A}$ or $AGO1^{S1001D}$ phospho-mutants (TABLE Nº 3, marked as 5/0.5). In the effort to understand why $AGO1^{S1001A}$ mutant does not respond to nitrate sufficiency as $AGO1^{S1001D}$ and wild-type plants, we studied the transcript accumulation pattern of genes responsive and non-responsive to nitrate availability.

A group of genes coding for a variety of protein functions as transferase (YLS5), serine protease (SBT3.3), heat shock protein family (Hsp70-2), and a calcium-binding protein, among others, were up-regulated under limiting nitrate concentration (0.5 Mm KNO_3) in the phospho-null mutant (FIG 17). These genes normally do not respond to the nitrate contrasting concentration in wild-type and the phospho-mimic plants, suggesting that the inability to phosphorylate AGO1 could alter transcript levels given the influence of the nitrate conditions.

Alternatively, we identified genes that do not respond to contrasting nitrate concentrations but were found to be down-regulated in the $AGO1^{S1001A}$ mutant under limited nitrate concentration (0.5 mM KNO_3) (FIG 18). It draws attention that TFs involved in the metabolism of hormones, amino acids, cell wall development, among others, appear from this list. Other interesting genes include enzyme activities such as invertase, transferase, hydrolase, and oxide-reductase (FIG 18B). These examples show that are not necessarily the genes that respond to nitrogen deficiency, those altered, but also other transcripts that do not change under these conditions.

Although under nitrogen-limiting conditions the differential accumulation of some transcripts could be explained given the inability to phosphorylate AGO1, there is another group of genes whose transcript levels are altered due to the concentration of nitrate in wild-type plants, but whose response is inhibited in $AGO1^{S1001A}$ or $AGO1^{S1001D}$ plants (FIG 19). This behavior is interesting because it would indicate that for a correct transcriptional response to N deficiency, the phosphorylation of AGO1 would be a necessary modification in *Arabidopsis thaliana*.

Taken together, these results suggest that plants that are subject to limiting concentrations of nitrate show transcriptional changes that are also conditioned by the phosphorylation status of AGO1.

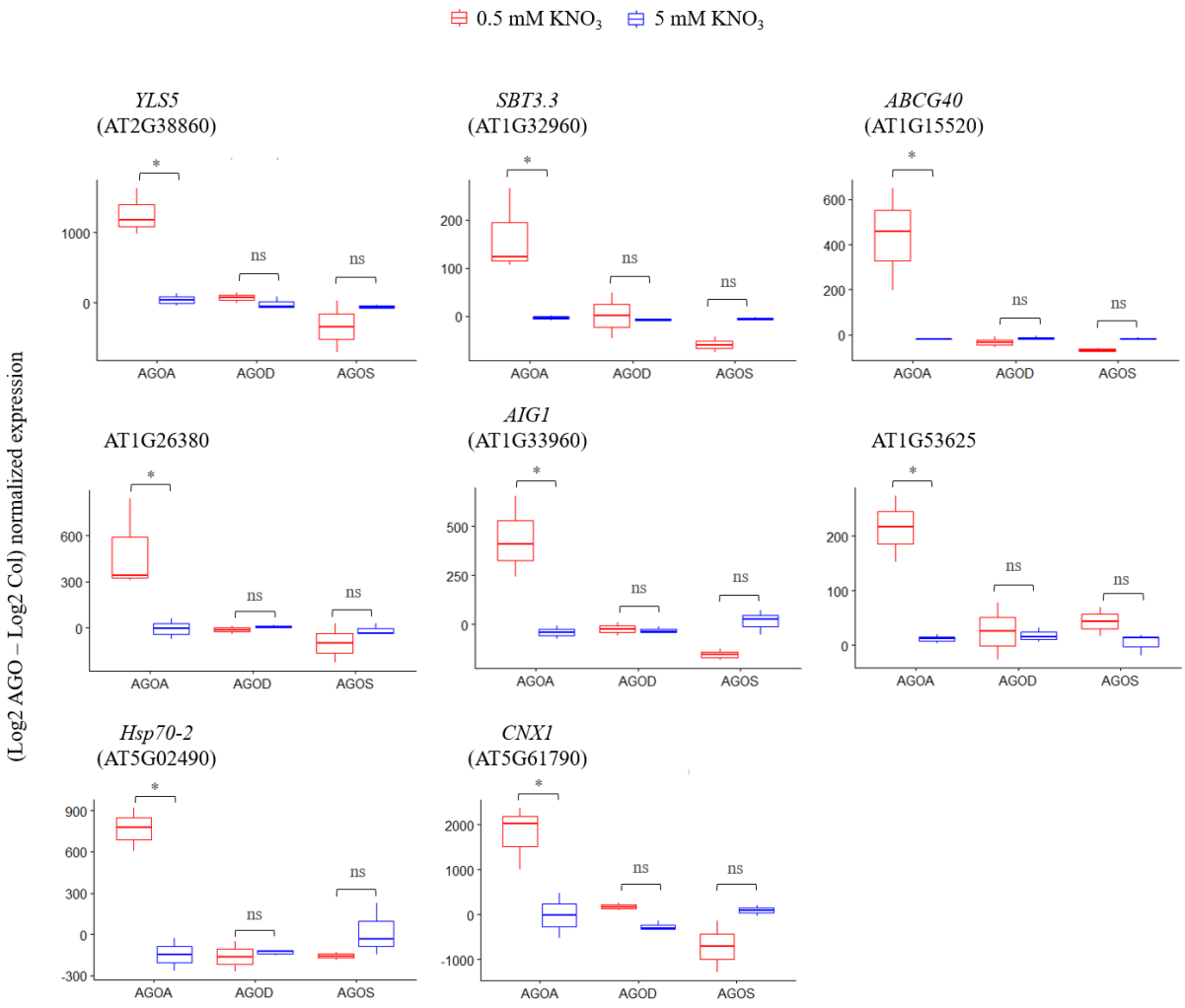


FIGURE 17. miRNA-targeted mRNAs are up-regulated under nitrate limiting concentration in the AGO1^{S1001A} mutant. Examples of the mRNA accumulation profiles of the predicted miRNA-targeted mRNAs that normally do not alter their mRNA accumulation under these experimental conditions. Reads subtraction of Col (Col-2) is showed for each transcript of the different AGO1 genotypes grown under limiting (0.5 mM KNO_3 in red) or sufficient (5 mM KNO_3, blue) nitrate concentrations. Asterisks indicate significative differences between treatments, $P < 0.01$. AGOA = AGO1^{S1001A}, AGOD = AGO1^{S1001D}, AGOS = AGO1^{S1001S} o wild type.

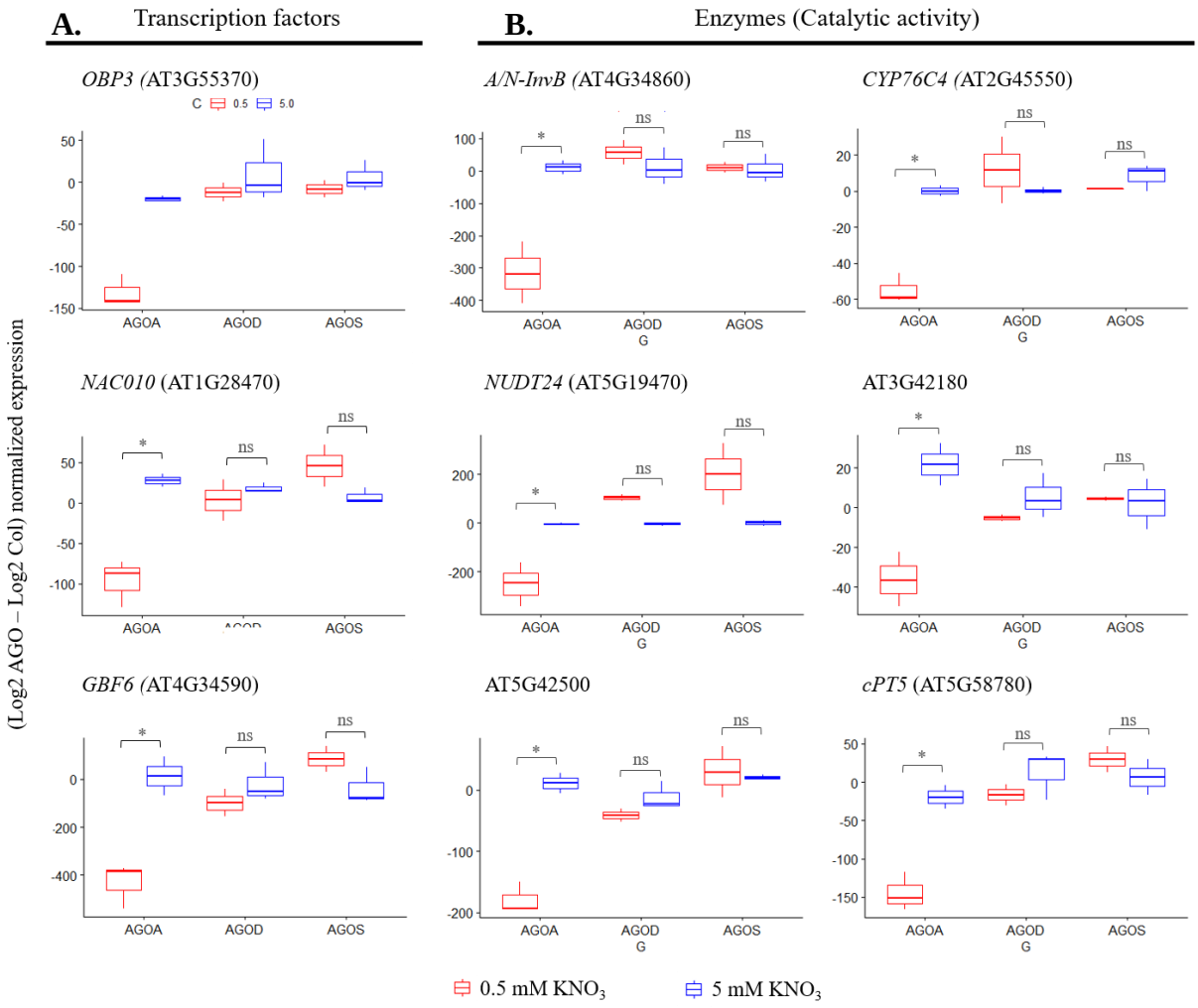


FIGURE 18. Alterations in the abundance of transcripts in mutant AGO1^{S1001A} under nitrate deficient conditions. Examples of mRNA accumulation profile of transcript genes coding for TFs **(A)** and enzymes **(B)**, targeted by miRNA. These genes normally do not respond to these experimental conditions in wild type (AGO^{S1001S}) but behave differentially in the *AGO1* mutants when grown in limiting nitrate. Reads subtraction of Col-2 is showed for each transcript of the different *AGO1* genotypes grown under limiting (0.5 mM KNO_3 in red) or sufficient (5 mM KNO_3, blue) nitrate concentrations. Asterisks indicate significative differences between treatments, P < 0.01. AGOA = AGO1^{S1001A}, AGOD = AGO1^{S1001D}, AGOS = AGO1^{S1001S} o wild type.

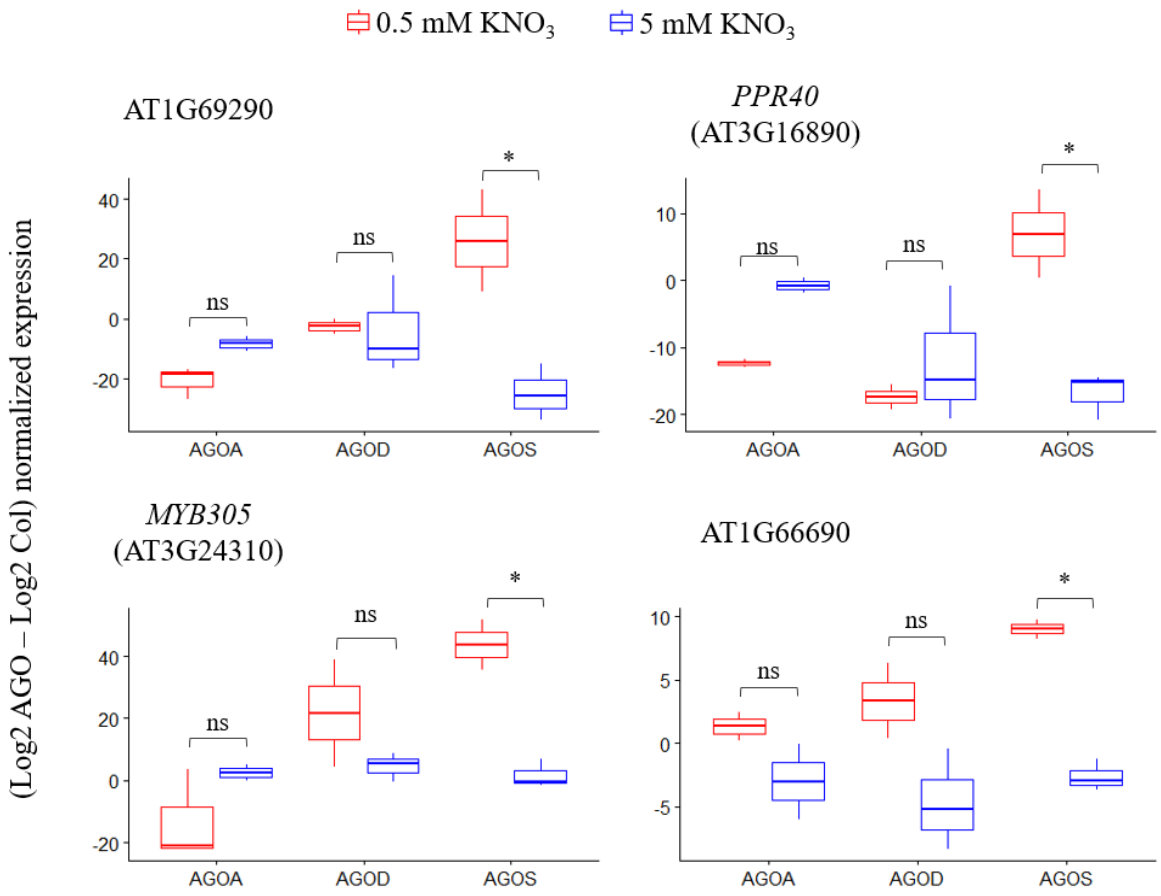


FIGURE 19. Normalized transcript abundance of genes whose response to the different nitrate concentration is lost in the AGO1 mutants. Examples of mRNA accumulation profile of genes targeted by miRNA that respond differentially to differentially nitrate concentration in wild-type but whose response is lost in the AGO1 phospho-mutants. These genes normally do respond to these experimental conditions in wild-type (AGO^{S1001S}) but behave differentially in the *AGO1* mutants when grown in limiting nitrate. Reads subtraction of Col-2 is showed for each transcript of the different *AGO1* genotypes grown under limiting (0.5 mM KNO_3 in red) or sufficient (5 mM KNO_3, blue) nitrate concentrations. Asterisks indicate significative differences between treatments, $P < 0.01$. AGOA = $AGO1^{S1001A}$, AGOD = $AGO1^{S1001D}$, AGOS = $AGO1^{S1001S}$ o wild type.

Although the analysis performed in this section focused on those genes targets of known miRNAs, it is worth mentioning that there is a list of genes whose transcript level is determined not only by the nitrogen condition, but also by the protein phosphorylation pattern, but they have not been described as substrates of known miRNAs.

5.4.6 mRNA cleavage (slicing activity) is altered in unphosphorylated AGO1.

AGO proteins possess an RNaseH-like endonuclease activity, the slicer activity (Song et al., 2004), that has been deeply studied, and attends at least three purposes: (1) It contributes to silencing by cleavage of target mRNA if guide strand and target mRNAs are base-paired around the middle of the duplex (Hutvágner and Zamore, 2002; Llave et al., 2002); (2) it can increase silencing responses by production of cleaved RNA fragments that are substrates for RNA-dependent RNA polymerases and hereafter produce new secondary siRNAs analogous to trans-acting siRNA (tasiRNA) biogenesis (Allen et al., 2005; Herr, 2005; Irvine et al., 2006; Qi et al., 2006); and (3) it facilitates strand separation of fully complementary small RNA duplexes by passenger strand cleavage (Matranga et al., 2005). Considering that the Ser1001 residue is located right next to the end of the PIWI domain, which possesses the catalytic activity, we asked whether phosphorylation site might impact AGO1 endonuclease activity. To evaluate so, we studied the slicer activity *in vitro* of both AGO1 phospho-mutants. Immunopurified wild-type, $AGO1^{S1001A}$, and $AGO1^{S1001D}$ were evaluated to examine the ability to mediate *in vitro* cleavage of the *PHV* transcript, target of the conserved miR165 (Prigge, 2004). By an *in vitro* reaction, *PHV* was ^{32}P-labbeled, and used as the substrate for the purified AGO1 complex.

We were able to detect a robust cleavage product after $AGO1^{S1001A}$, $AGO1^{S1001D}$, and the wild-type AGO1 acted over the *PHV* RNA target (FIG 20).

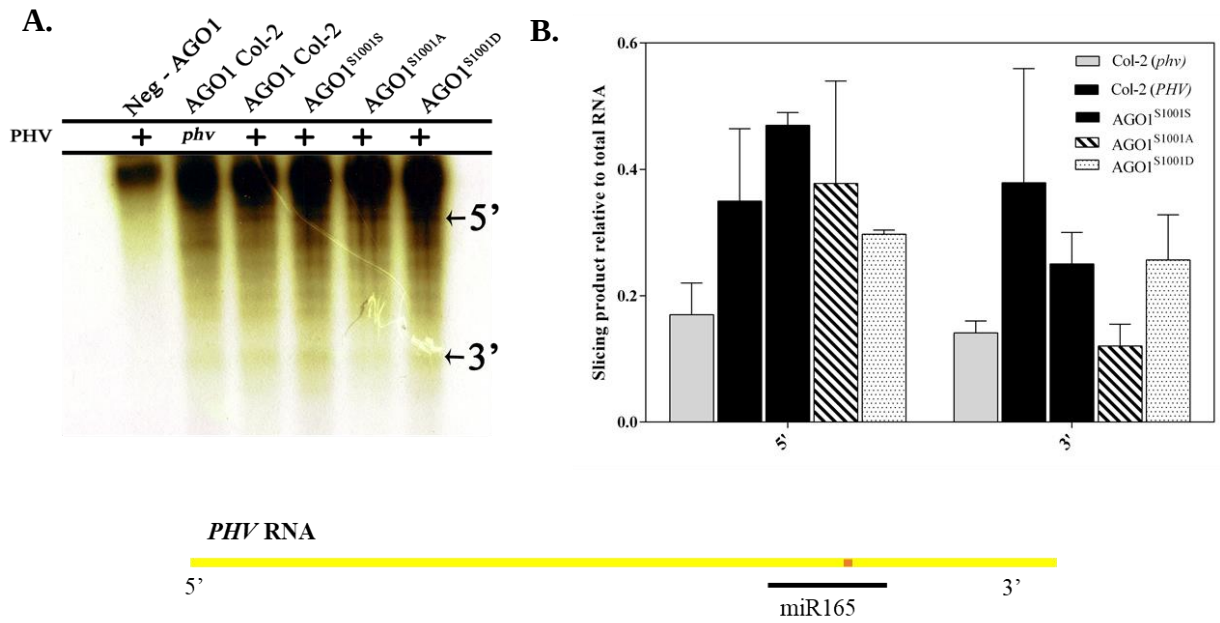


FIGURE 20. AGO1 Slicer activity. **A.** *In vitro*-labeled WT *PHV* and mutant *phv* target RNAs were incubated with immunoprecipitated from wild-type (Col-2 and AGO1^{S1001S}), AGO1^{S1001A}, and AGO1^{S1001D} plants. The black arrows indicate the predicted 3' *PHV* RNA cleavage products. AGO1 was immunoprecipitated from 1 g of seedlings. Twenty microliters of the eluate were assembled in a reaction containing ^{32}P-UTP labeled *in vitro* transcribed target RNA. WT *PHV* target templates for *in vitro* transcription were generated by PCR on *A. thaliana* cDNA containing the *miR165/166* target site. After 120 min at 25°C – 30°C, RNA was extracted from the reaction. The labeled RNA was separated on 7 M Urea/ 8% polyacrylamide gel (Miniprotean II system, Bio-Rad) and detected in the gel after exposure to X-film. **B.** Cleavage product quantification. 5' and 3' RNA products were quantified using ImageJ software for two independent replicates.

However, comparing to total product recovered, reaction involving the AGO1^{S1001A} mutant displayed less than half of the 3' transcript product. Although this result could be explained by the stability of the excised RNA given experimental conditions, the observed result could explain the altered function of AGO1 phospho-mutants, and therefore, the hypomorphic phenotype of the phospho-null mutant, in which no total activity of AGO1 is abolished.

6. DISCUSSION

6.1 AGO1 phospho-site is needed to complete plant development properly.

Exhaustive studies indicate that plant small RNA biogenesis and action are processes firmly controlled. PTMs, especially phosphorylation, causes fast and transient protein modulation in a wide range of cellular processes and external stimuli responses. Here, we investigated the morphological and molecular consequences of mutating the well-conserved Ser1001 residue of AGO1, on plant responses to different nitrogen conditions. To date, its been shown both, post-transcriptional and post-translational processes fine tune AGO1 contents. However, there is no evidence of phosphorylation as a PTM that controls AGO1 activity, stability or other functional mechanism.

Here, we showed that, although phospho-mutant plants expressed normal levels of *AGO1* mRNA and protein, characteristics such as plant size, flowering time and fertility are affected especially when the AGO1 protein is unable to be phosphorylated (FIG 3, FIG 4 and FIG 6).

Developmental defects of AGO1 phospho-mutants range from small rosette size, delay in the transition from juvenile to adult seedling, flower and siliques development, and fertility reduction. It's been shown that there are at least two types of *ago1* mutants. The ones showing very strong developmental defects, most of them are sterile *in vitro* or in soil (*ago1-1* to *ago1-24* and *ago1-*

36) (Bohmert et al., 1998; Fagard et al., 2000; Baumberger and Baulcombe, 2005), and a second group that are fertile and show limited developmental defects such as *ago1-25* and *ago1-27* alleles (Morel et al., 2002). Since it is experimentally difficult to evaluate developmental characteristic in the first group, we added the *ago1-27* allele to some of our analysis. As it was previously reported, we observed that *ago1-27* allele initiates flowering 10 to 12 days after wild type plants, following the strongest delay when compared to the AGO1^{S1001A} and AGO1^{S1001D} mutants. Considering that many reports have shown that weak *ago1* alleles display, for instance, delay in organ production, allele-specific defects in organ polarity, reduced fertility, narrow organs and serrated leaves (Fagard et al., 2000; Morel et al., 2002; Boutet et al., 2003; Kidner and Martienssen, 2004) our findings account as these phenotypic effects can be considered as weak to medium strength.

As we mentioned earlier, miRNAs play crucial roles in almost all aspects of normal plant growth and development. Developmental phase transitions are not the exception. AGO1 phospho-null mutant showed a delay in abaxial trichome formation, which means the duration of the juvenile phase is longer than wild type (FIG 4). In our time-course gene expression analysis, we observed reduced levels of SPL3 mRNA, which is consistent with the delay in abaxial trichome formation, and with what has been observed in plants with reduced levels of *SPL* regulatory factors due to overexpression of the *MIR156* gene (Wu et al., 2009; Xu et al., 2016). The miR156/SPL module represents a major regulatory axis for the promotion of flowering in the aging pathway (Wang et al., 2009). Normally, miR156 accumulates through vegetative growth, but it peaks during the early juvenile phase and decreases gradually during the subsequent vegetative phases (Wang et al., 2009). Other studies have shown that central components of the miRNA biogenesis machinery, HYL1, and SE, control the accumulation of miR156. For instance, *hly1* mutants have reduced levels of miR156 leading the upregulation of SPL3 gene (Li et al., 2012a). It is interesting

to notice that, differently to SPL9, SPL3 does not directly alter miR172 nor its targets accumulation, which was confirmed in our results (FIG 4), suggesting a possible selectivity of the phosphorylated AGO1. Considering our data, we propose that at least part of the aging pathway components is deregulated in the phospho-null plants. However, more experiments are needed to determine the degree of involvement of this modification in the juvenile to adult transition, as well as flowering time. The study of the expression of the main regulators of the developmental phase transitions at the tissue and cell-specific level would inform if the phosphorylation of AGO1 has any impact on a specific regulator or would be affecting the process in an integral way (many regulators). In addition, it would be interesting to evaluate the degree of alteration of the target-mRNAs association to the RISC complex, as well as to determine the subcellular localization of the protein in the different stages of *Arabidopsis* development and organs.

Our findings strongly suggest that additionally to the presence of the protein, the Ser1001 residue is required to have a full-active protein. According to our knowledge, to date, the function that AGO1 phosphorylation could have on RISC complex assembly or its direct activity is unknown. However, several studies have deciphered the impact of numerous phosphorylation residues on the animal orthologous AGO2, some of which have been suggested to regulate miRNA maturation (Shen et al., 2013), RISC assembly and function (Rüdel et al., 2011), slicer activity (Zeng et al., 2008; Horman et al., 2013; Lopez-Orozco et al., 2015), protein-protein interaction (Horman et al., 2013; Jee and Lai, 2014), cellular localization (Zeng et al., 2008) and shifting activity from slicer to translational repression (Horman et al., 2013). Considering these antecedents together with the fact that the Ser1001 residue is conserved across the plant kingdom and the

evidence showed here, it makes sense to think that part of the functionality of the AGO1 protein is given by the phosphorylation pattern or integrity of the Ser1001 residue.

6.2 The machinery behind miRNA activities is important for the correct response and development of plants subjected to nitrate stress/stimulus.

Considering the multiple regulatory layers in which AGO1 is involved, our main objective was to determine the link between the nitrate-dependent phosphorylation pattern of AGO1, and the function of the miRNAs during the normal response to nitrate of *Arabidopsis*. We found AGO1 phospho-mutants displayed significant but not severe changes in shoot and root development under normal or sufficient nitrate concentration (FIG 6 and 8). Interestingly, the $AGO1^{S1001A}$ mutant was unable to respond to the contrasting nitrate as wild-type and the phospho-mimic plants ($AGO1^{S1001D}$). Concerning the biological functions of miRNA machinery in biotic or abiotic stress, many miRNA pathway mutants exhibit compromised responses to stress. It's been reported that AGO1 mutant *ago1-27* show acid abscisic (ABA) hypersensitivity and drought tolerance (Morel et al., 2002; Navarro et al., 2008; Li et al., 2012b).

Previous reports have found *Arabidopsis* AGO1 been phosphorylated under several environmental stimuli. Phospho-proteomics performed under hormones (ABA) treatments (Wang et al., 2013a), biotic stress (flg22) (Rayapuram et al., 2017), ionizing radiation (Roitinger et al., 2015), osmotic stress (Xue et al., 2013), and nitrate starvation/supply treatments (Wang et al., 2013b) have detected the phospho-peptide corresponding to AGO1. AGO1 differential phosphorylation or any functional aspect related to external cues has not been described yet. On the other hand, animal AGO2 phosphorylation has been linked to stress responses (Shen et al., 2013). For instance, Tyr393-phosphorylation suppresses the maturation of specific tumor-

suppressor-like miRNAs in response to hypoxic stress, which correlates with inferior overall survival in breast cancer patients (Shen et al., 2013).

Together to our observations, the actual knowledge points to AGO1 phosphorylation as a modification that possible regulates AGO1 function, and therefore the miRNAs action when the plant faces disadvantageous conditions.

Nitrate serves not only as a form of nitrogen in the soil but also as a potent signaling molecule that regulates numerous processes including gene expression and root architecture. During the early response, in which de novo protein synthesis does not occur, nitrate triggers phosphorylation pattern changes in *Arabidopsis* roots. Vega et al., (in process) in their recent work observed that nitrate leads to reduced levels of phosphorylated AGO1 after 20 minutes nitrate treatments. In this context, a constantly dephosphorylated protein would mimic the nitrate treatment, as if nitrate was present, which is consistent with the shorter primary root of the phospho-null plant (FIG 6). Considering a different growth condition, we registered a different response. Under contrasting nitrate concentration, we observed that the AGO1^{S1001D} mutant developed longer primary root than wild-type and the phospho-null mutant (FIG 3C and 3D). Under such conditions, phospho-null plants showed no responses to sufficient nitrate as wild-type. Phospho-null showed developmental defects as they were grown under limiting nitrate (FIG 9). Indeed, Sorin et al. (2005) showed that the hypomorphic alleles *ago1-33*, *ago1-32* and *ago1-34*, showed longer primary root length under normal growth conditions, while the strong mutant *ago1-3* root is almost three times longer than the wild-type (for the same hypocotyl size) when germinating in the dark. Given the importance of the root system and what we could observe from AGO1^{S1001D} and AGO1^{S1001A} genotypes (FIG 3, 7 and 9), the regulation of root system architecture has more than miRNAs and their targets as mediators. The impact of altering AGO1, one of the main components of the machinery

responsible for the miRNA role, demonstrated that we need to understand the coordination between exogenous factors and endogenous signal pathways to predict some responses to environmental stimuli during root organogenesis.

At the beginning of this investigation, we addressed two main questions: (1) Is the AGO1 phosphorylatable residue relevant during the molecular and cellular function of AGO1? and (2) Do mutant plants respond as wild type to nitrate availability of *Arabidopsis* plants? Even though AGO1 mutant plants showed an altered response to nitrate, the mechanism behind the phosphorylation remain hidden. Concerning the molecular relevance of AGO1 phosphorylation, miRNA abundance and their association with AGO1 were evaluated. Our analysis showed that when Ser1001 residue was changed, practically unaffected miRNA metabolism and RISC-binding was observed (FIG 10). This is consistent with what it's been reported for the hypomorphic *ago1* alleles. They generally do not exhibit reduced miRNA accumulation, but show increased miRNA target accumulation (Vaucheret et al., 2004; Baumberger and Baulcombe, 2005; Carbonell et al., 2012). Thus, loss of Ser1001phophorylation does not impair miRNA biogenesis or loading into AGO1 (Vaucheret et al., 2004; Baumberger and Baulcombe, 2005; Carbonell et al., 2012).

Conventional understanding holds that miRNA loading into AGO proteins occurs in the cytoplasm. However, a studied model shows nuclear miRNA loading and nuclear export of loaded AGO1 occurs (Bologna et al., 2018; Liu et al., 2018). It's been observed that chromatin bound AGO1 positively regulates the level of gene expression (Liu et al., 2018). To determine whether AGO1 phosphorylation could alter seedling transcriptome, mRNA sequencing of Col-2 and AGO1-Ser1001 mutants analysis reveals a group of genes differentially expressed given the nitrate concentration and AGO1 mutation. It was interesting to observe that under different nitrate concentration, a few genes showed altered expression in the $AGO1^{S1001D}$ mutant, but almost four

hundred genes respond differentially in the AGO1^{S1001A} mutant (relative to wild type) when nitrate was poorly supplied. The previous results showed that more profound changes were observed in AGO1^{S1001A} than in AGO1^{S1001D}, which supports the fact that AGO1^{S1001A} plant exhibited more severe phenotype defects than the AGO1^{S1001D} plant (FIG 3, FIG 13). In order to get more insights about AGO1 functions besides post-transcriptionally regulation, the study of AGO1 cellular localization was also considered in this work. Although the experimental work is ongoing, and the results are unavailable yet, the methodology used will allow us to observed wild-type and mutant AGO1 expression to determine to what extent the alteration of the nature of the Ser1001 residue alters the subcellular localization of the protein.

Although with these findings we are not able to determine the precise function of the AGO1 phosphorylation during the response of *Arabidopsis* to nitrate, according to our knowledge, this would be the first time that a PTM of a central protein working on small RNA metabolism was linked to the molecular response of *Arabidopsis* to nitrate availability.

6.3 Future challenges

The identification of many players in the nitrate response and the miRNA action has shed light on the latest decades. However, key challenges remain concerning the relationships and precise biochemical contributions of the core players' modification and small RNA activity. At the subcellular level, it is of interest to investigate how the phosphorylation or other PTMs modulate processes as growth and development in response to nitrogen availability. One side involves the impact of nitrate as a signal that modifies the phosphoproteome and the other, how the dynamics of phosphorylation are necessary for the rapid and efficient responses triggered by plants when faced environmental stimulus.

Knowing that AGO1 functions are many and varied, the challenge of pointing to one affected task becomes complex. That is, at what stage of the small RNAs metabolism where AGO1 participates should we aim to continue this research? We have already seen that neither the abundance nor the union of the miRNAs are significantly altered given the inability to phosphorylate AGO1. We also observed that the transcriptional response of the phospho-null mutant differs from that observed in the wild type or the phospho-mimic plant when faced with deficient N conditions. With these data, we get the idea that the phosphorylation of AGO1 is not only relevant for the correct timing in the development, but that it would also have a role in the adaptive response to unfavorable nutritional conditions. This is the first report indicating that the residue Ser1001 is relevant for plant development and, it is probably implicated in target-miRNA processing and gene silencing.

Our knowledge about miRNAs and its roles in the nitrate signaling pathway is still limited, that's why future work will unravel the impact of PTMs on the miRNA activity machinery, as well as in its consequences in plant sensing and responses to different environmental cues. One of our transcriptomic analysis showed that phospho-deficient plants displayed deregulated genes as part of the biological function "stress response" which was overrepresented. This makes sense since AGO1 is one of the major targets of viral silencing suppressors, allowing a virus to attack by inhibiting the plant silencing machinery (Baumberger et al., 2007; Chiu et al., 2010; Derrien et al., 2012).

Since this work unravels the role and some mechanistic function behind AGO1 serine phosphorylation, there are still many open questions regarding this layer of regulation affecting AGO1 in *Arabidopsis thaliana.*

Starting with the assumption that part of AGO1 function relies on the phosphorylation status, some question arises: To what degree, AGO1 single phosphorylation alters its function? Are there other phospho-sites that need to be studied? As it was shown in animals (Golden et al., 2017; Quévillon Huberdeau et al., 2017; Collingridge et al., 2018), is the combinatory effect of several phosphorylation sites what affects AGO1 function profoundly? How many different states of the protein exist depending on phosphorylation?

Regarding AGO1 activities, many other questions could be discussed: Is the protein conformational state and/or cellular localization altered by phosphorylation? Are then, protein-protein o protein-nucleic acid interactions affected due to AGO1 phosphorylation status? For example, there is good evidence that AGO proteins exist in two different states depending on whether the 3′-end of its guide strand is fully base-paired to a target mRNA because base-pairing to the 3′part is accompanied by a release from the PAZ domain (Zander et al., 2014). Thus, is not unreasonable to hypothesize that, *in vivo*, phospho-deficient AGO1 mutants might be locked in a single conformational state, potentially not capable to perform all functions of the wild-type protein. However, these possibilities depend on where the modification occurs and how protein folding, or interaction might be affected.

A recent investigation in mammalian AGO2 showed a phosphorylation cluster located at the end of the PIWI domain which does not affect miRNA loading or AGO2 localization but affects mRNA binding.

On the other hand, our analysis showed that AGO1 preferentially bound to sRNAs that are 21 nucleotides in length, which is consistent with the known AGO1-binding preference (Mi et al., 2008; Liu et al., 2018) and most likely produced by DCL1 with assistance from HYL1 (Liu et al., 2018). Recently, it was described that AGO1 is not limited to the cytoplasm only, it's been found

that AGO1 in the nucleus regulate gene expression through chromatin association. This association was previously shown to be impaired in the HYL1-knockout mutant (*hyl1-2*) or upon modification of the HYL1 phosphorylation status (in the *cpl1-7* mutant) (Dolata et al., 2016). It would be interesting to evaluate no only $AGO1^{S1001A}$ and $AGO1^{S1001D}$ cell localization pattern but also the production and function of other small RNAs species such as siRNA, tasiRNAs, and hcRNAs.

Plants must constantly deal with external stimuli that modulate internal pathways which translate into fast and efficient dynamic developmental changes. That is why the only increase in expression levels of miRNAs is not fast enough to mediate modulation of the local proteome to drive the miRNA-dependent gene silencing in responses to the nitrate availability. While our results are revealing, they open several profound questions whose answers will require time and effort that will undoubtedly be worth investing. How phosphorylation (or other PTMs) plays as a critical modification for protein plasticity and dynamic is a question that needs to be addressed to get insights into the very first decisive steps driven by cells that face a diversity of environmental cues.

7. CONCLUSION

In summary, what we found during this investigation can be listed as follow: (1) AGO1 Ser1001 residue phosphorylation is altered in Arabidopsis roots facing a nitrate pulse; (2) The mutation of this residue leads growth defects, especially when it is replaced by a neutral amino acid such alanine; (3) *Arabidopsis* developmental responses to the nitrate status it's abnormal in plants expressing AGO1^{S1001A} which indicate that the integrity of the Ser1001 residue is key for normal growth and plant responses to environmental stimuli such nitrate availability. Moreover, regarding to the molecular basis, we can conclude: (1) accumulation of miRNAs in phosphor-defective mutants is normal, contrary to ago1 null plants; (2) loading of miRNAs in slicer-deficient AGO1 is very similar to AGO1^{S1001S} (3) slicer-deficient AGO1 can target PHV transcripts for production of cleavage products (4) the two different mutants tested behaved differently according to development and transcriptional responses and (5) *Arabidopsis* AGO1^{S1001A} showed more severe morphological and molecular phenotype than wild type and the phosphor-mimic mutant plants. Nevertheless, other distinctive characteristics of AGO1 protein might be evaluated the association/dissociation from target mRNAs, protein localization, protein-protein association, and translational repression, all functions exerted or directly influenced by AGO1.

The study of these features could help to understand how phosphorylation can change the properties of the protein.

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