UNIVERSITY OF CALIFORNIA SAN DIEGO

Stress-inducible and ubiquitous knockdown of Clade A Type 2C protein phosphatases (PP2Cs) for increased drought tolerance in *A.thaliana* and *B.napus*

A thesis submitted in partial satisfaction of the requirements of the degree Master of Science

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by

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The Thesis of Alexandre Francois Miaule is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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Co-Chair

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Chair

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ABRE	ABA-responsive element
ANOVA	Analysis of variance
CRISPR-Cas9	Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9
DOG1	Delay of germination 1
DRE	Drought-responsive element
MS	Murashige and Skoog media
PP2C	Clade A Type 2C protein phosphatases
PYR/PYL/RCAR	Pyrobactin resistance 1/ PYR1-like/ Regulatory components of ABA receptors
Rd29a	Resistant to desiccation 29a
Rd29b	Resistant to desiccation 29a
ROP	Rho of Plants
RopGEF	Rop guanine nucleotide exchange factor

ABSTRACT OF THE THESIS

Stress-inducible and ubiquitous knockdown of Clade A Type 2C protein phosphatases (PP2Cs) for increased drought tolerance in *A.thaliana* and *B.napus*

by

Alexandre Francois Miaule

Master of Science in Biology

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Professor Julian I. Schroeder, Chair
Professor Alisa Huffaker, Co-Chair

Plants are continuously subjected to various abiotic stresses. In agricultural settings, one of the most destructive abiotic stresses is drought, which is actively becoming a pressing and relevant issue due to climate change and increasing water scarcity. Drought stress has a heavy impact on crop yields and overall crop health, presenting a problem with major economic ramifications. As such, development of novel methods for drought-tolerant crops presents a solution for farming in increasingly arid conditions.

In this study, known negative regulators of the drought signaling pathway were knocked down using RNAi to generate increasingly drought tolerant plants for both *Arabidopsis thaliana* and *Brassica napus*. Previous studies have shown that knocking out Clade A Type 2C protein phosphatases (PP2Cs), which are negative regulators in the drought signaling pathway, resulted in enhanced drought tolerance, but has come at the cost of decreased growth in non-drought conditions. As a mechanism for enhancing drought tolerance without compromising yield in well-watered conditions, RNAi was utilized to knockdown *PP2Cs* instead of knocking them out. Therefore, we generated stress-inducible and constitutive knockdowns of PP2Cs in *A.thaliana* and stress-inducible knockdowns of PP2Cs in *B.napus*. We found that both stress-inducible and constitutive *A. thaliana* knockdown lines allow enhanced ABA responsiveness in germination. A well-controlled low moisture assay was developed that resulted in demonstration of drought tolerance in stress-inducible and ubiquitous knockdown lines in *A.thaliana*. Some *B.napus* stress-inducible lines show enhanced ABA responsiveness. However, enhanced drought tolerance of these lines is still uncertain. This research demonstrates the effectiveness of knocking down specific negative regulators in the drought signaling pathway in order to generate more drought-tolerant plants.

INTRODUCTION:

As sessile organisms, plants are under constant environmental pressure, such as drought, heavy metal content, extreme temperatures or high salinity conditions. These abiotic stresses have detrimental effects on plant growth and overall fitness, and limit agricultural production (Mickelbart, Hasegawa, & Bailey-Serres, 2015; Mittler, 2006). Notably, drought stands as one of the major abiotic stresses impacting agriculture, classified by the FAO as the most impactful natural disaster in the last four decades (http://www.fao.org/land-water/water/drought/en/). Global temperatures have consistently increased over the last 80 years, and it is expected that extreme climate events, especially drought, will become more prevalent (Dai, 2013; Diffenbaugh et al., 2017). With increasing global demand in water, along with progressively more arid conditions, farming becomes increasingly difficult (Carrão, Naumann, & Barbosa, 2016; Sivakumar et al., 2014). New approaches for improving drought resistance are needed. In order to alleviate this problem, farming has turned towards modern biotechnology to genetically engineer more drought tolerant crops (Georges et al., 2009; Quan, Shang, Zhang, Zhao, & Zhang, 2004; Xiao et al., 2009).

Plants possess many strategies to mitigate environmental pressures, but one of the major drought stress modulators in plants are stomata. Stomata are pores on the leaf surfaces that gate CO2 uptake for photosynthesis along with water loss through transpiration (Jarvis & Morison, 1981; Jones, 1998). Drought stress induces stomatal closure in plants, a process which is mediated by specialized cells called guard cells (Schroeder, Kwak, & Allen, 2001). Guard cells respond to

drought through depolarization of the plasma membrane of the guard cell, causing ion water to be released from the cells, which results in a loss in guard cell turgidity, which in turn closes the stomata and prevents transpiration (Munemasa et al., 2015; Waadt et al., 2015). Furthermore, some plants induce dormancy at the seed stage under arid conditions as a means to prevent germination under unfavorable conditions (Huang et al., 2017). Drought tolerant plants have also been shown to enter metabolically dormant states by limiting plant and root growth as a drought resistance mechanism (Zheng et al., 2016).

While plants employ many means to mitigate water stress, a central phytohormone is coordinates drought avoidance strategies. Abscisic acid (ABA) is a carotenoid-based phytohormone which plays a central role in responses to abiotic stresses, and most notably is heavily implicated in drought responses in plants (Cutler, Rodriguez, Finkelstein, & Abrams, 2010). Biosynthesis of ABA is triggered as a result of drought stress, causing a signaling cascade culminating in stomatal closure. When drought is perceived, ABA is synthesized and the ABA concentration increases. ABA binds to receptors in the Pyrobactin resistance 1 (PYR)/ PYR1 Like (PYL)/Regulatory components of ABA receptors (RCAR) family, creating a trimeric complex with the clade A type 2C protein phosphatase family (Ma et al., 2009; Melcher et al., 2009; S. Park et al., 2010; S. Y. Park et al., 2009). Formation of this trimeric complex inhibits the action of PP2Cs on SNF1-related kinases (SnRK2). SnRK2 are responsible for activation of downstream transcription factors and ion channels which are involved in stomatal closure (Umezawa, Yoshida, Maruyama, Yamaguchi-Shinozaki, & Shinozaki, 2004; Waadt et al., 2015). Without the presence of ABA, PP2Cs inactivate SnrK2s via dephosphorylation, thus preventing ABA-related stomatal closure (Melcher et al., 2009; Munemasa et al., 2015).

Regulation of germination and primary root elongation in plants is also dependent on the

action of PP2Cs. Increases in endogenous ABA have been shown to prevent PP2C activity and induce seed dormancy (Nishimura et al., 2007). Notably, genes key for the induction of seed dormancy, such as Delay of Germination 1 (DOG1), interact with PP2Cs as a means to regulate ABA related responsiveness (Nishimura et al., 2018). ABA-related seed dormancy and inhibition of root elongation constitute additional drought resistance mechanisms by which plants prevent growth in arid conditions.

Structural and biochemical analysis of the drought signaling pathway has revealed nine PP2Cs that are involved in the drought signaling pathway. Efforts to generate more drought tolerant plants have led to the knockout of PP2Cs as means to increase water use efficiency. Drought tolerance in plants can be measured in a variety of manners, such as water use efficiency, reduced leaf transpiration or as reduced biomass penalties in periods of water scarcity (Kantar, Lucas, & Budak, 2011). Single PP2C knockouts have limited effects of the ABA responsiveness in plants (Santiago et al., 2009). However, multiple knockouts of PP2Cs show enhanced drought tolerance in plants. Double PP2C knockouts of *hab1-1 abi1-2* show enhanced drought tolerance, along with enhanced ABA responsiveness in germination (Saez et al., 2006). Drought tolerant phenotypes are further accentuated with additional knockouts, with the quadruple loss of function mutant showing severe delay of germination in ABA conditions (*abi1-abi2-hab1-pp2ca*) (Li, Waadt, & Schroeder, 2016). The loss of function mutants showed higher water use efficiency, but also penalties in their biomass by reducing the overall growth of the plant in non- drought conditions. The balance between enhanced drought tolerance due to PP2C knockouts and reduced biomass in well-watered conditions poses a debate over whether knockouts in crop plants may be applicable in agriculture.

RNAi has been demonstrated as a useful means by which to target multiple genes in the same family without completely suppressing the expression of the targeted gene (Bezanilla, Perroud, Pan, Klueh, & Quatrano, 2005). RNAi has been used extensively as a means to

circumvent otherwise lethal knockouts, or avoid severe phenotypes (Banno, Ikeda, Niu, & Chua, 2001; Gordon & Waterhouse, 2007). Furthermore, RNAi has successfully been able to be used to generate more drought tolerant plants (Ouyang et al., 2010; Zhou et al., 2012) . By utilizing RNAi as a means to knock down PP2Cs, we seek to alleviate the growth penalties associated with full loss of function of these genes. Furthermore, RNAi allows the added benefit of controlling the expression of the knockdown based on the promoter type we utilize. Using this methodology in a model and a crop background we seek to determine the effects of constitutive and stress-inducible knockdown PP2Cs.

Identification of the viral promoter 35S has given plant biologists a useful approach towards inducing constitutive changes to plants (Banno et al., 2001; KAY, CHAN, DALY, & MCPHERSON, 1987). Constitutive approaches towards knockdown of certain genes through the 35S promoter have been shown to be effective in generating drought tolerant plants (Nelson et al., 2007; Umezawa et al., 2004). Investigation of the ubiquitous 35S promoter, would be interesting in studying the effects of constitutively knocking down PP2Cs, and an intriguing transgene to compare to rd29a-induced knockdown of PP2Cs.

In order to achieve enhanced drought tolerance while maintaining maximal biomass levels in non- drought conditions knockdown over loss of function knockout mechanisms must be explored, specifically stress-inducible knockdowns may yield promising results. Many abiotic stress-inducible genes, such as *RD29B* have been shown to be responsive to drought as a result of increased levels of endogenous ABA (Nakashima et al., 2006). Genes such as *RD29B* are expressed in the presence of drought and ABA due to the presence of cis-acting elements called ABA-responsive elements (ABRE) in their promoter region. The homologous gene *RD29A* was shown to be more responsive to drought, while maintaining minimum responsiveness to ABA

(Msanne, Lin, Stone, & Awada, 2011a). Identification of multiple Drought-responsive elements (DRE) in the promoter region of *RD29A* makes it more responsive to drought than its homologue *RD29B* (Narusaka et al., 2003). Identification of *RD29A* as a strong drought-induced gene, makes it an interesting candidate for investigating use of the *RD29A* promoter as a means to manipulate gene expression in response to drought.

Species from the *Brassicacea* family have long been the subject of study as model organisms for drought, specifically the model organism *Arabidopsis thaliana* has been instrumental in elucidating the drought signaling pathway (Meinke, Cherry, Dean, Rounsley, & Koornneef, 1998; Salekdeh, Reynolds, Bennett, & Boyer, 2009). The extensive research done on *A.thaliana* for investigation of the drought signaling pathway has led to development of novel drought avoidance strategies in closely related species (Dalal, Tayal, Chinnusamy, & Bansal, 2009). *Brassica napus*, a member of the *Brassicacea* family, is an allotetraploid species generated from the hybridization of the diploid species *Brassica rapa* and *Brassica oleracea*. *B.napus* is an important oil crop, ranking third in oil production among crop plants after oil palm and palm kernels, and taking up 35 million hectares in cultivation space globally (Friedt, Tu, & Fu, 2018). Due to their enormous relevance in agriculture, the effects of abiotic stresses, such as drought, on yield and growth of B.napus are of great economic value. The genomic similarities between *A.thaliana* and *B.napus* allow a bridge between the extensive literature found for *A.thaliana* and a crop plant such as *B.napus* (Wang et al., 2011).

In our study, we generated constructs allowing stress-inducible and constitutive knockdowns of PP2Cs in *A.thaliana* and stress inducible knockdowns of PP2Cs in *B.napus*. RNAi technology was used to target negative regulators of the ABA signaling pathway without requiring gene knockouts. As higher order *PP2C* knockouts are associated with enhanced drought

resistance, but also decreased well-watered biomass, it was hypothesized that drought inducible knockdowns of PP2Cs would enhance drought resistance without inhibiting growth under non-drought conditions. We determined that lines for both stress-inducible and constitutive knockdown of PP2Cs in *A.thaliana* showed enhanced drought tolerance without any penalties in non-drought conditions. The stress inducible and constitutive knockdown lines in *A.thaliana* showed enhanced seed dormancy under ABA conditions, while only the constitutive knockdown lines showing increased ABA-associated root growth stunting. Finally, the few *B.napus* stress-inducible knockdown lines that we have obtained show some ABA related enhanced seed dormancy, but no clear or consistent enhanced drought tolerance. Our findings demonstrate the effectiveness of using RNAi to knock down PP2Cs under drought inducible and constitutive promoters as a means to generate more drought tolerant plants, while circumventing the growth inhibitions associated with higher order *PP2C* knockouts.

MATERIALS AND METHODS:

Plant Material and Growth conditions

We acquired the T-DNA insertion line (*SALK_130229*) for Guanine exchange factor (*GEF2*) through the Arabidopsis Biological resource Center (ABRC) at Ohio State University. A homozygous knockout was identified using the protocol described by (Alonso & Stepanova, 2003), and primers were designed with the aid of http://signal.salk.edu/tdnaprimers.2.html; sequence provided (Table 1). Both *A.thaliana* and *B.napus* seeds were sterilized using a solution of 7% bleach and 0.05% Tween-20. Seeds were then cold-treated for 4 days with no light at 4°C. Seeds were sown on ½ Murashige and Skoog (MS) (Murashige & Skoog, 1962) adjusted to a pH of 5.6- 5.8 using KOH, and Agar concentration of 1% for 7 days then transferred to soil and grown in a growth room at humidity (60 ± 2% RH) with 12-h light/12- h dark at 150 μmol $m^{-2}s^{-1}$ at 21°C.

Plant transformation

The *p35S::RNAi-PP2C* and *prd29a::RNAi-PP2C* constructs were transformed into *A .thaliana* Columbia (Col-0) plants using *Agrobacterium tumefaciens* clones containing the plasmids using floral dipping method (Logemann, Birkenbihl, Ülker, & Somssich, 2006). Transgenics were selected for in ½ MS and 50 ng/μl Kanamycin. Transgenic lines selected for Kanamycin resistance were genotyped using the following primers (Table 1). Homozygous transgenic lines were selected for in non-segregating lines on Kanamycin plates in the T3 generation.

Table 1: Primers used for Genotyping

Primer used	Gene	Purpose
ATCTAAGCAATGGAGCCACTG	*GEF2* (AT1G01700)	Forward genomic primer
AACATCCTGCAAACCACAAAC	*GEF2* (AT1G01700)	Reverse genotyping primer
ATTTTGCCGATTTCGGAAC	*GEF2* (AT1G01700)	T-DNA binding primer
CAATAGCAGCCAGTCCCTTC	Type 2C Clade A Protein Phosphatases RNAi	Forward genotyping primer
AGACAATCGGCTGCTCTGAT	Type 2C Clade A Protein Phosphatases RNAi	Reverse genotyping primer

Germination assay

Sterilized *A.thaliana* seeds were cold treated for four days, then grown on ½ MS (±1.5 μM ABA) for 9 days. For *B.napus* germination assays, seeds were cold treated for four days then grown ½ MS (with or without 2 μM ABA) for 5 days post-sterilization. Plates were incubated in the same growth conditions as Pater, Mullen, McKay, & Schroeder, (2017). Photos were taken and cotyledon emergence was scored using Image J, after 9 days for *A. thaliana*, and 5 days for *B. napus* (Rueden et al., 2017)

Root Growth assay

Sterilized *A. thaliana* seeds were cold treated for 4 days then grown in ½ MS for 5 days, and seedlings with similar primary root length were transferred to ½ MS (with or without 20 μM ABA) for 7 days of growth. Plates were grown vertically in the same growth conditions as Pater, Mullen, McKay, & Schroeder, (2017). Primary root length was assessed using ImageJ (Rueden et al., 2017). Statistically significant differences between root lengths were assessed using one-way ANOVA followed by Tukey's multiple comparisons test.

Drought assay

A mixture of dry potting soil and vermiculite (4:1) was prepared in 4.5 inch pots, and dry weight was adjusted to the nearest weight for each pot. Pots were bottom watered and allowed to sit overnight, excess water was discarded the next day. One week old seedlings were transferred from ½ MS to the pots with one seedling per quadrant approximately 1 cm from the pot edges. Seedlings were allowed to grow for two more weeks and separated into two groups, either drought or non- drought. All pots were weighed and soil moisture content was measured using a METER EC-5 soil moisture sensor every other day. Non-drought pot's weight was adjusted after every measurement to be identical to the heaviest pot on day one by adding water. Drought pots were not adjusted for the first two weeks after separating groups. After 2 weeks, drought pots were now adjusted to their group's heaviest pot weight as found on day 14 of drought treatment. After 1 week of maintaining low moisture levels, seedlings in both conditions were cut at the soil surface and were weighed for fresh shoot weight. Representative pictures for the final day of the experiment were adjusted to increase contrast from background using ImageJ (Rueden et al., 2017). Statistically significant differences between fresh weights were assessed using one-way ANOVA followed by Tukey's multiple comparisons test.

Fritted Clay drought assay

Pre-moistened "Profile Porous Ceramic (PPC) "Greens Grade soil" soil was used to fill to the top of 4.5 inch pots. Trays with the pots were then filled 2 cm up the height of the pot in water and covered with clear domes overnight. Excess water was removed and replaced the next day to the same amount. This process was repeated 3 times in order to reduce dust and contaminants in the soil. After this process, the tray was supplied with a ½ strength Hoagland media 2cm uo the height of the pot (Hoagland & Martin, 1923) overnight . One week old *B.napus* seedlings were transferred from ½ MS to the pots, with one seedling per quadrant, approximately 1.5 cm from the pot edges. Seedlings were allowed to grow for one week, and separated into two groups, either drought or non-drought. Pots during this period were bottom watered one hour every other day with ½ strength Hoagland media up to 2 cm up the height of the pot. Plants were grown in the same growth conditions as Pater, Mullen, McKay, & Schroeder, (2017). Growth conditions and watering habits were maintained for non-drought group pots, Drought group pots were no longer watered for 29 days, and bottom watered with ½ strength Hoagland media up to 2 cm up the height of the pot for one hour the day before the end of the assay. All pots' soil moisture content was measured using a METER EC-5 soil moisture sensor every other day. After 30 days of drought, seedlings in both conditions were cut at the soil surface and were weighed for fresh shoot weight. Statistically significant differences between fresh weights were assessed using one-way ANOVA followed by Tukey's multiple comparisons test.

RNA extraction and qPCR

Seeds were germinated on ½ MS for 5 days and were transferred to incubate in plates with ½ MS (with or without 20 μM ABA) for 0, 2 and 4 hours. RNA from 6-8 seedlings was then extracted at each time point and condition using the SpectrumTm Plant Total RNA kit (Sigma

Aldrich) following manufacturer's instructions. Concentration was then measured using a nanodrop, 1μg of RNA per sample was treated with Turbo DNA-*free* Kit (NEB), and cDNA was synthesized using first strand cDNA synthesis kit (GE healthcare) as per manufacturer's instructions. Synthesized cDNA was diluted 3 times, and 1 ul was used per PCR reaction as template. qPCR was performed on a BioRad CFX96 qPCR System using SYBR Select Master Mix (Applied Biosystem) with gene-specific primers (Table 2).

Table 2: Primers used for qPCR

Gene name	Forward primer	Reverse primer
PDF2 (At3G22480)	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGCTTGG
HAB1 (At1G72770)	GTCATGGAGGCCATAAGGTTGC	ACCTGCCTACCCTCTCCTGTATTC
ABI1 (At4G26080)	CATGTCGAGATCCATTGGCGAT AG	ACTCTCTTCACAGCCGTCACTT C
PP2CA (At3G11410)	TCCTCTCTCCGTAGATCACAAG CC	ACTCCAAGAACCCTAGCTCCAT CC
HAB2 (At1g17550)	TGAAGGGATGAGTCCAAGTCT CC	TGGCAATAGTCAGCAACCTGA GC
Type 2C Clade A Protein phosphatase RNAi	TGTACGGCGTGACTTCCATC	TCCTCCGCTAAAGCCAAGTG

RESULTS:

Generation of RNAi constructs targeting Type 2C Clade A Protein Phosphatases

Clade A Type 2C Protein phosphatases are highly conserved negative regulators of the drought signaling pathway in plants (Furihata et al., 2006; Ma et al., 2009; Rodrigues et al., 2013). RNAi targeting of Clade A Type 2C Protein Phosphatase homologues in *A. thaliana* and *B. napus* (ABI1, ABI2, HAB1, PP2CA) started in our lab by Dianne Pater using the Brassica Database (Cheng et al., 2011). The region targeted by the RNAi is a 400 bp highly conserved catalytic region common to both *A. thaliana* and *B. napus* Clade A Type 2C Protein Phosphatases (Figure 1). The RNAi construct for *B. napus* was designed under the inducible promoter *prd29a*, a strong abiotic stress inducible promoter which is especially responsive to drought due to the presence of multiple drought responsive elements (DRE) (Msanne et al., 2011a; Narusaka et al., 2003). This drought-inducible promoter was used to investigate the effects of knocking down PP2Cs under drought stress, while maintaining wild-type expected ABA response levels under non-drought conditions. In *A. thaliana*, RNAi PP2C expression was also driven by *prd29a*, to investigate whether the construct would confer a drought resistant phenotype in both a crop and a closely related model organism. Additionally, with the same RNAi sequence we generated a construct under the viral ubiquitous promoter *p35S*, which is predicted to allow for whole plant constitutive knockdown of Clade A PP2Cs.

In *A.thaliana* 10 independent lines containing *35S*-driven PP2C RNAi (p35S::*RNAi-PP2C*) construct and 8 independent lines containing *rd29a*-driven PP2C RNAi (prd29a::*RNAi-PP2C*) constructs have been generated. Dianne Pater in our lab previously generated 12 independent lines containing *rd29a*-driven PP2C RNAi constructs for *B.napus*. Later, I generated 3 homozygous

independent lines of both *35S* and *rd29a*-driven PP2C RNAi constructs for *A.thaliana* by selecting for T3 non-segregating lines.

Effect of stress inducible and constitutive knockdown of Type 2C Clade A Protein Phosphatases in ABA for A. thaliana

To investigate the effects of our knockdown lines under both stress-inducible and constitutive promoters in *A.thaliana*, we compared seed germination of our transgenic lines to wild-type in 1.5 μM ABA containing plates, along with the ABA hypersensitive triple knockout mutant *hab1-1 abi1-2 pp2ca-1* as a control (Li et al., 2016; Santiago et al., 2009) (Figure 2 and Figure 3). A consistent ABA hypersensitive germination phenotype was found in all three independent homozygous lines for both constructs (Figure 2 and 3). The delay of germination seems to be weaker under the *rd29a* promoter as compared to the constitutive *35S* promoter. (Figures 2 and 3).

To further investigate this phenotype, I looked into ABA-regulation of root growth. For this, we transferred seedlings to 20 μM ABA plates and measured primary root elongation for 9 days (Figure 4). While the lines for the *35S*-driven PP2C knockdown construct showed enhanced ABA responsiveness at the seedling stage as shown through ABA inhibition of root elongation, *rd29a*- driven PP2C knockdown lines show wild-type like ABA responsiveness (Figure 4 A and C).

rd29a and 35S-meditated PP2C knockdown lines are more drought tolerant

To examine if knockdown of PP2Cs would affect plant water use efficiency in a drought assay, we established a low moisture drought assay (Figure 5). In order to mitigate differences in soil from pot to pot, and establish a more uniform environment for studying water use efficiency

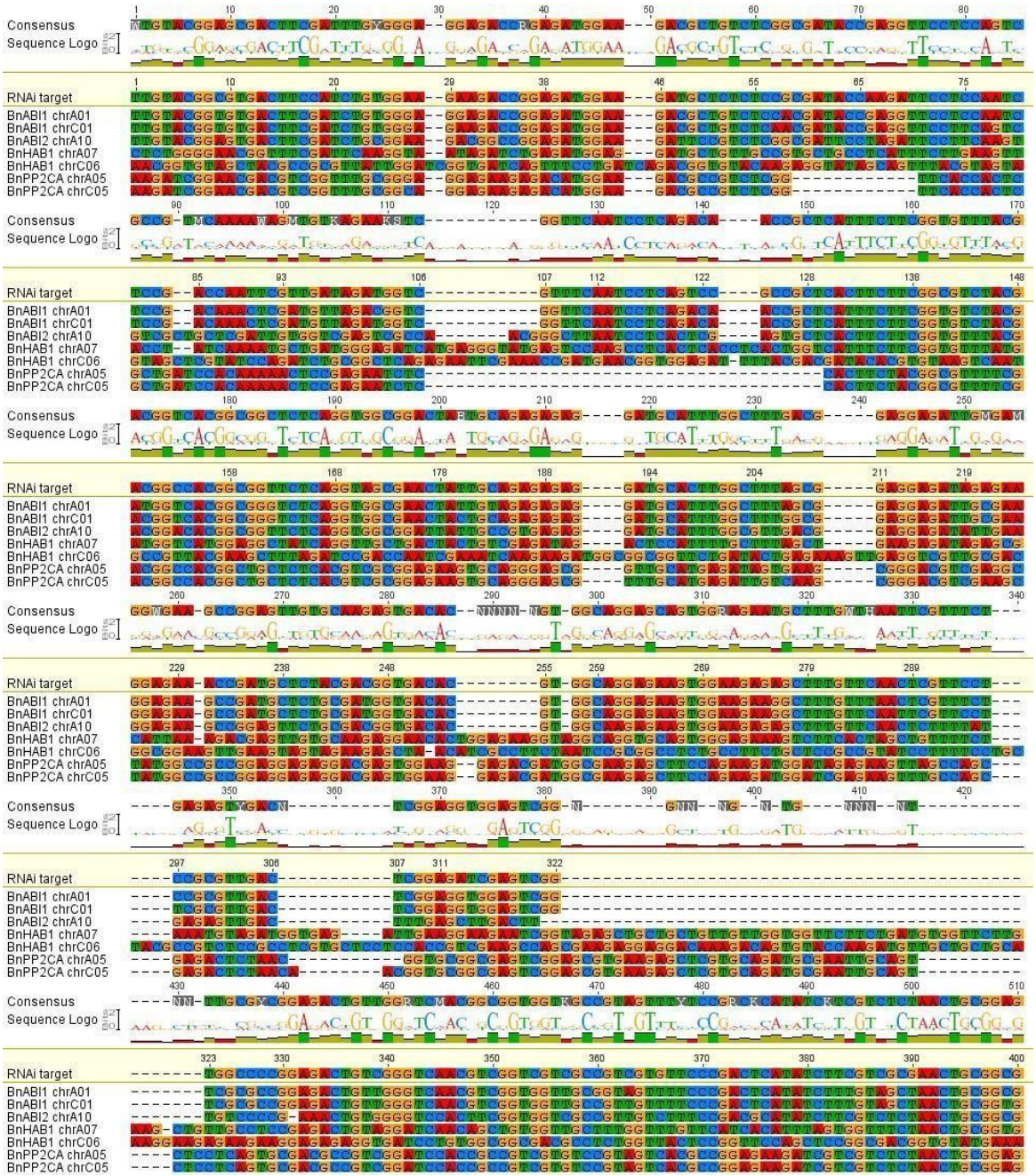
between genotypes, we adjusted water weight (Figure 5B). By monitoring the weight of pots and moisture levels of the pots, we circumvented issues of variability in water availability for our plants, along with establishing uniform stress conditions. We found that after a period of 6 weeks in non-drought conditions, there was no growth penalty in *rd29a* and *35S* mediated PP2C knockdown mutants. (Figure 5C and D), indicating that our constructs posed no measurable effect in well-watered conditions under the imposed conditions. After 3 weeks of water-limiting conditions, we found that our ABA hypersensitive knockout mutant *hab1-1 abi1-2 pp2ca-1*, and our *rd29a* and *35S* mediated PP2C knockdown mutants showed no significant growth penalty as compared to their non-drought counterparts (Figure 5 C and E). However, wild type plants show a significant decrease in total biomass as compared to their non-drought equivalents (Figure C and E) (significance as assessed by one-way ANOVA followed Tukey post-test). Our transgenic lines for both *rd29a* and *35S*-driven PP2C knockdowns show enhanced drought tolerance without compromising growth in non-drought conditions.

Effect of stress inducible knockdown of Type 2C Clade A Protein Phosphatases in ABA for B.napus

ABA inhibition of seed germination was tested in *rd29a*-driven PP2C knockdown *B.napus* transgenic lines in 2 µM containing ABA plates (Figure 6). Out of the three lines I tested, it was observed that two of them had enhanced ABA hypersensitivity, while the third line was shown to have wild-type like ABA responsiveness in germination (Figure 6). One of our ABA hypersensitive line (line 9), showed a consistent delay of germination as assessed by cotyledon emergence in non-ABA conditions that was observed in three independent experiments (Figure 5).

To examine if knockdown of PP2Cs would affect plant water use efficiency in a drought assay, we established a drought assay (Figure 7). In order to mitigate differences in water

availability from pot to pot, and establish a more uniform environment for studying water use efficiency between genotypes we looked into using fritted clay, which has been demonstrated to dry down more evenly than soil. (Dianne Pater, unpublished (2017)). We measured soil moisture levels to confirm effective drydown (Figure 7A). In non-drought conditions, our *B.napus rd29a* mediated PP2C knockdown mutants show some variability in growth, with one line (line 17) showing some growth penalty (Figure 7B). After 30 days of drought, we found that two of our lines (line 10 and 17) may be more drought tolerant, however more replicates are needed to confirm the phenotype (Figure 7B). Our transgenic lines show signs of being more drought tolerant, but more replicates need to be run to establish a clear phenotype.



B.

Figure 1: Comparison of RNAi target sequence (first line) to six published *B.napus* PP2C genes *ABI1, ABI2, HAB1,* and *PP2CA*. A. RNAi sequence target sequence had highest consensus with *ABI1* and *ABI2*. Source Dianne Pater, Schroeder lab (unpublished) B. RNAi target sequence.

RNAi target sequence

TTGTACGGCGTGACTTCCATCTGTGGAAGAAGACCGGAGATGGAAGATGCTCTCTCC
GCGATACCAAGATTCCTCCAATCTCCGACCAATTCGTTGATAGATGGTCGTTTCAAT
CCTCAGTCCGCCGCTCACTTCTTCGGCGTCTACGACGGCCACGGCGGTTCTCAGGTA
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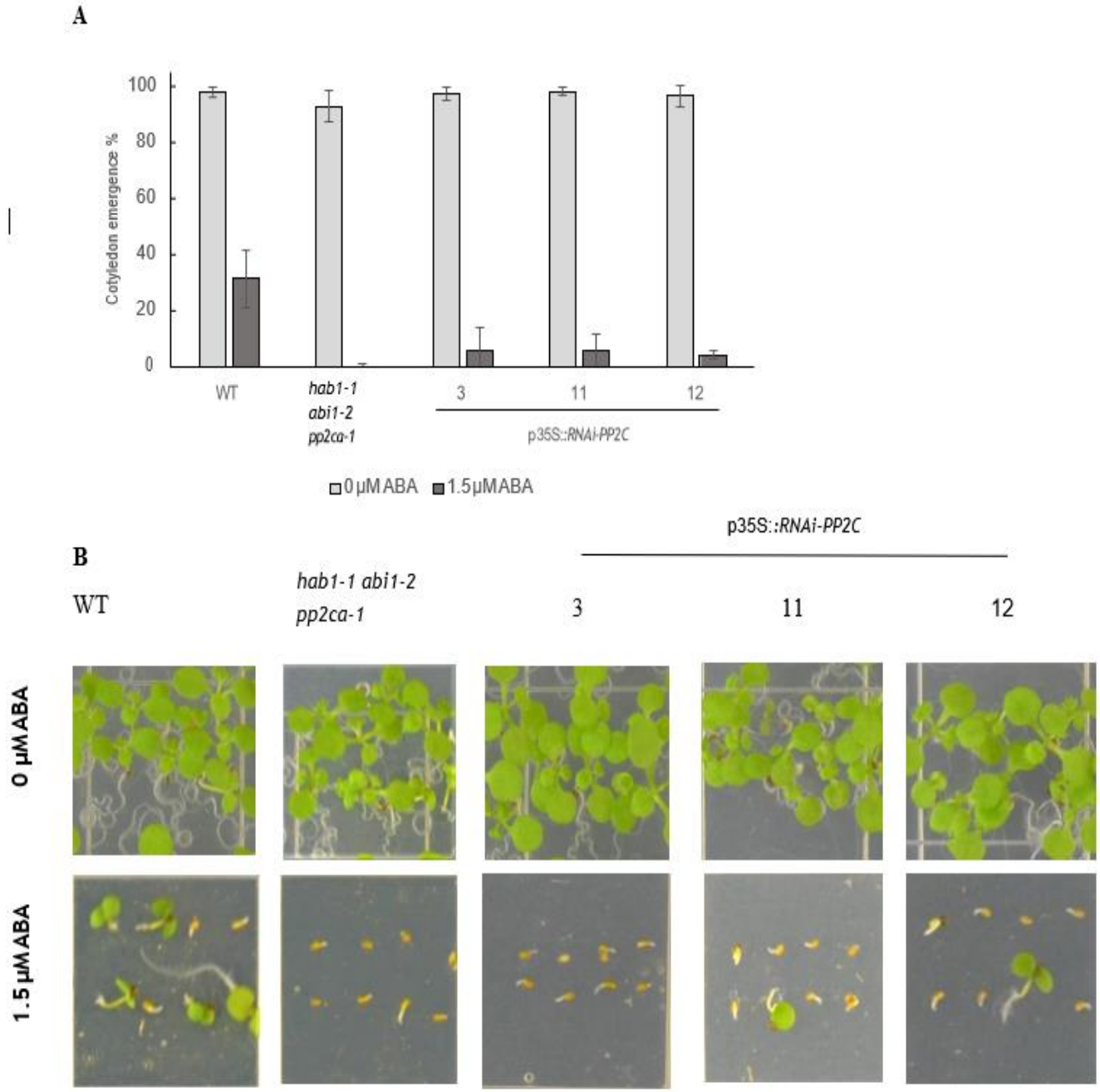
GAAACCGATGCTC



<u>Figure 2:</u> 35S-mediated Clade A PP2C knockdown lines show enhanced ABA hypersensitivity as measured by cotyledon emergence percentage. A Cotyledon emergence of WT, *pp2c4xko* and 35S mediated Clade A PP2C knockdown lines in 0 µM ABA and 1.5 µM ABA 9 days after stratification. Errors bars represent standard deviation.(n= 3 replicates, each of 40-50 seeds, analyzed per experiment in each genotype) **B** Representative images of WT, *hab1-1 abi1-2 pp2ca-1* and 35S-mediated Clade A PP2C knockdown lines in both 0 and 1.5 µM ABA conditions after 9 days

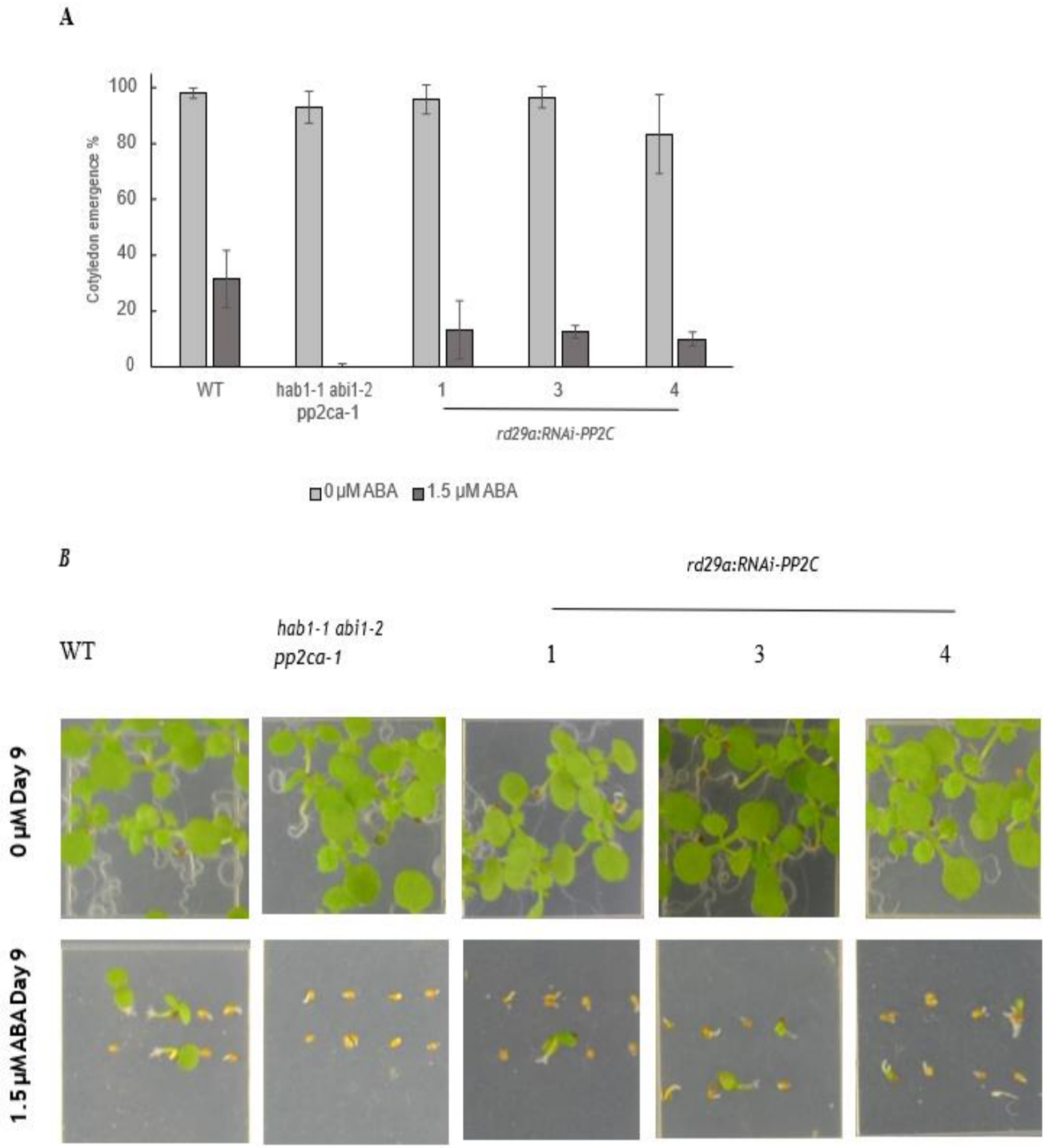


Figure 3: Rd29a-mediated Clade A PP2C knockdown lines show enhanced ABA hypersensitivity as measured by cotyledon emergence percentage. **A** Cotyledon emergence of WT, *pp2c4xko* and rd29a mediated Clade A PP2C knockdown lines in 0 µM ABA and 1.5 µM ABA 9 days after stratification. Errors bars represent standard deviation.(n= 3 replicates, each of 40-50 seeds, analyzed per experiment in each genotype) **B** Representative images of WT, *hab1-1 abi1-2 pp2ca-1* and Rd29a mediated Clade A PP2C knockdown lines in both 0 and 1.5 µM ABA conditions after 9 days.

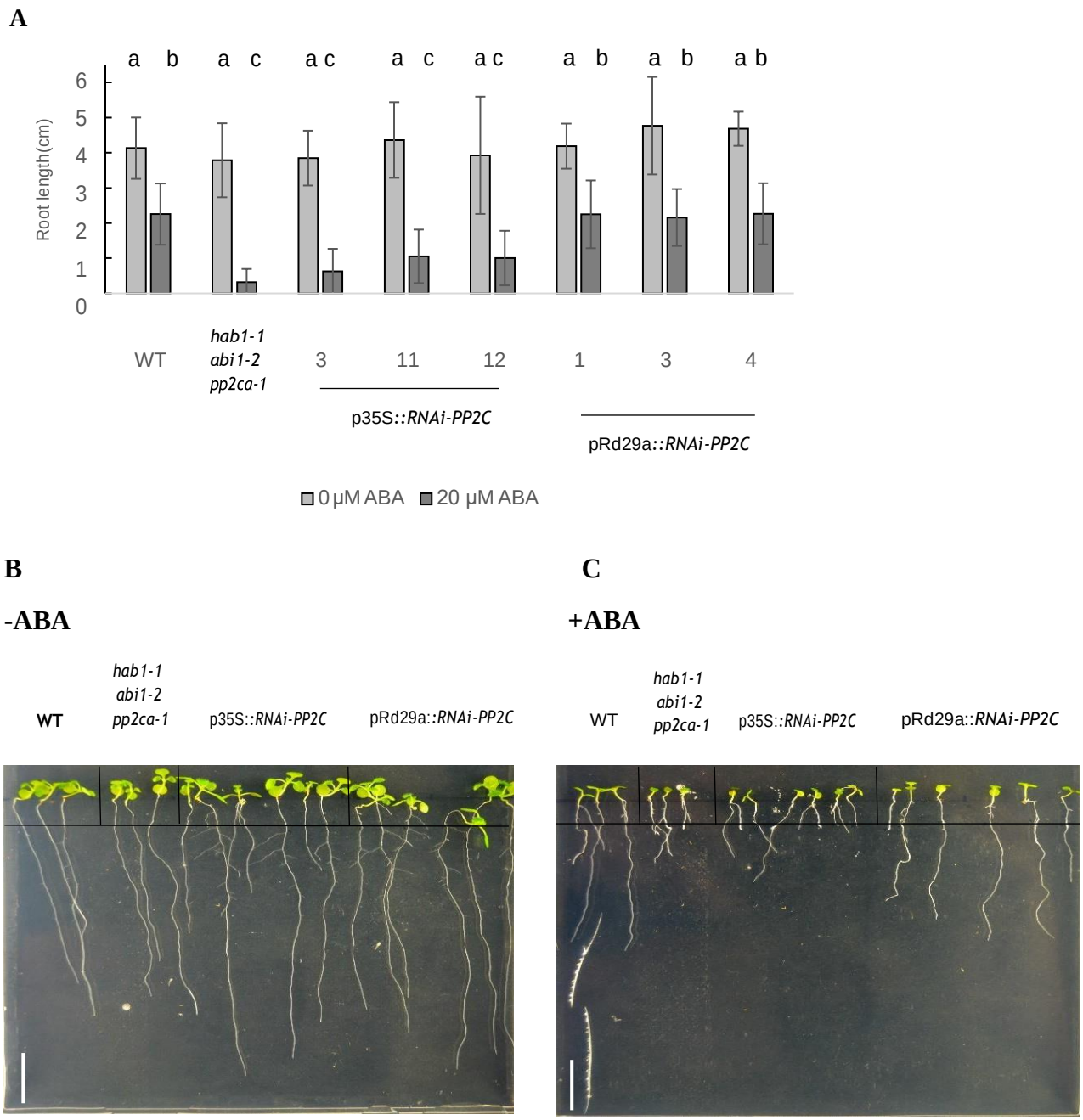


Figure 4: 35S-mediated PP2C knockdown lines show enhanced ABA hypersensitivity during root elongation, while R29a mediated PP2C knockdown lines show no hypersensitivity to ABA. A. Root elongation quantification on 35S- and Rd29a-mediated PP2C knockdown lines as compared to WT and *hab1-1 abi1-2 pp2ca-1* in 0 µM ABA and 20 µM ABA after 7 days of treatment.(n= 6 replicates of 3-4 seedlings) letters represents differing significance as assessed by one-way ANOVA followed Tukey post-test. **B**. Representative images of root lengths of 11 day old WT, *hab1-1 abi1-2 pp2ca-1*, 35S mediated PP2C knockdown and rd29a mediated PP2C knockdown ½ MS conditions, seedlings were grown for 4 days in ½ MS, then transferred to ½ MS plates for 7 days. **C**. Representative images of root lengths of WT, *hab1-1 abi1-2 pp2ca-1*, mediated PP2C knockdown and rd29a-mediated PP2C knockdown ½ MS conditions, seedlings were grown for 4 days in ½ MS and transferred to 20 µM ABA plates for 7 days.(n= 6 replicates of 3-4 seedlings analyzed per genotype per experiment) letters represents differing significance as assessed by one-way ANOVA followed Tukey post-test. Scale bar represents 1 cm

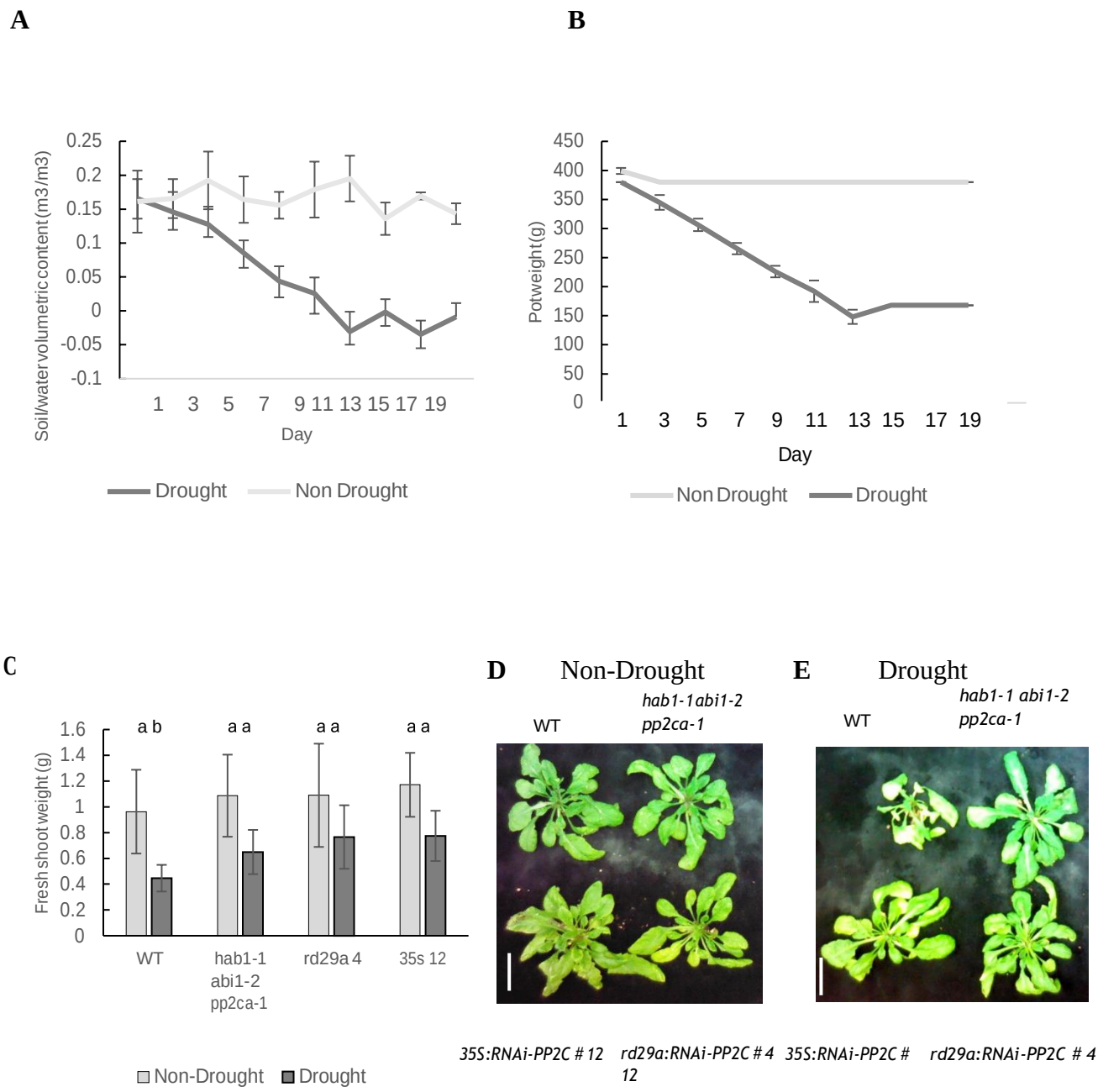


Figure 5: Enhanced drought tolerance in both drought-inducible and ubiquitous mediated knockdown of Clade A Protein Phosphatases. **A** Soil moisture levels in pots under drought and non-drought conditions over 19 days (n=8 pots). **B** Pot weight in both drought and non- drought conditions over 19 days (n=8 pots).**C** Fresh weight of WT, *hab1-1 abi1-2 pp2ca-1* , *rd29a 4*, and *35S 12* under drought and non-drought conditions (n=7 individual plants). **D** Representative picture of 5 week old WT, *hab1-1 abi1-2 pp2ca-1* , *rd29a 4*, and *35S 12* under non-drought conditions grown in the same pot, excised and placed on a black background. **E** Representative picture of 5 week old WT, *hab1-1 abi1-2 pp2ca-1* , *rd29a 4*, and *35S 12* under drought conditions after 19 days grown in the same pot, excised and placed on a black background. Significance assessed using ANOVA, letters represent differing significance. Scale bar represents 1cm

A

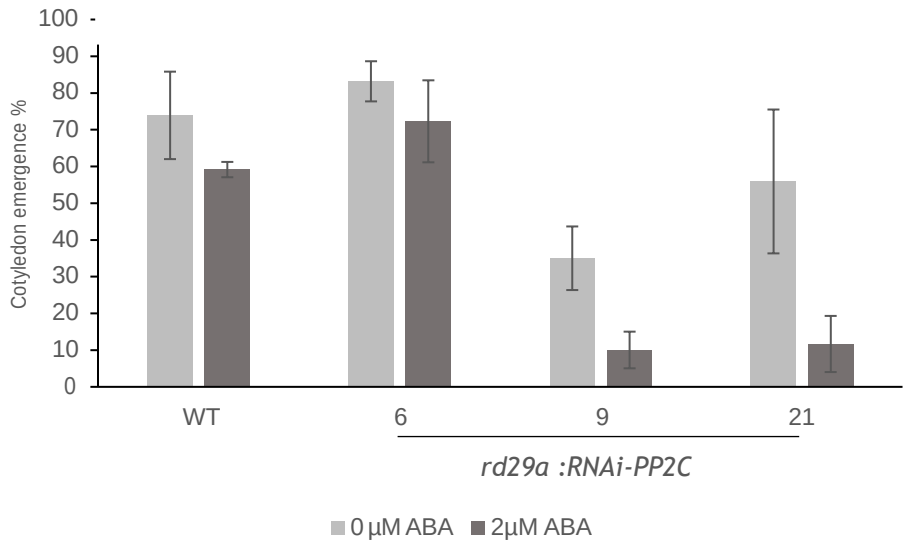
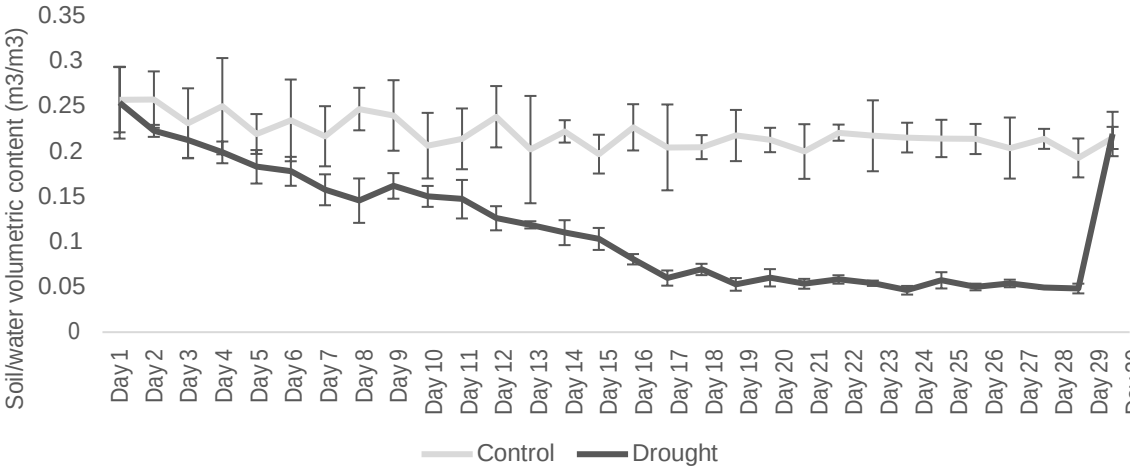
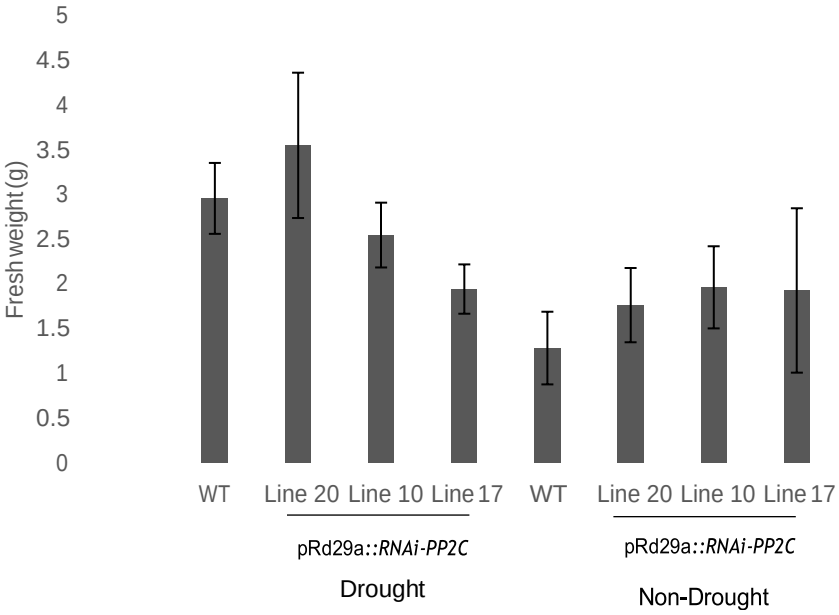


Figure 6: Rd29a-mediated PP2C knockdown lines in *B.napus* show some enhanced ABA hypersensitivity as measured by cotyledon emergence rate . **A** Cotyledon emergence rate of *A napus* rd29a mediated lines 6, 21, 9 and WT in ½ MS conditions. (n=3 biological replicates, 20-30 seeds per plate). Error bars represent standard deviation.

A



B



<u>Figure 7:</u> Rd29a-mediated PP2C knockdown lines in *B.napus* show some enhanced drought tolerance. A. Soil moisture levels in pots under drought and non-drought conditions over 30 days (n=6 pots). **B.** Fresh weight of *B. napus* rd29a-mediated lines 6, 21, 9 and WT under drought and non-drought conditions (n=6 individual plants)

DISCUSSION:

Effects of rd29a and 35S-mediated Clade A Type 2C Protein Phosphatase knockdown in A.thaliana

While the RNAi design for the knocking down of Clade A Type 2C Protein phosphatases (PP2C) was intended for *B.napus*, a crop plant in the same family as *A.thaliana* (Figure 1), the modelling was done through PP2C orthologues from *B.napus* and *A.thaliana*. Due to the similarities between *A.thaliana* and *B.napus* PP2Cs, we looked to test both species for drought-related phenotypes at different growth stages. Here, we describe the drought and ABA responsiveness of our *A.thaliana* mutants.

We found that both drought-inducible and constitutive PP2C knockdown lines yielded enhanced ABA responsiveness during germination, with constitutive knockdown lines showing increased germination stunting as compared to drought-inducible (Figure 2 and 3). Similarly, *35S*-mediated *PP2C* knockdown lines again showed higher ABA responsiveness through limitation of primary root growth in the presence of ABA as compared to the wild-type levels exhibited by the *rd29a*- mediated knockdown lines (Figure 4). The decreased ABA response in germination and root elongation of *rd29a*-mediated knockdown and *35S*-mediated knockdown lines is consistent across each of the respective three lines tested. Abiotic stress-inducible promoters such as *rd29a* or *rd29b* have cis-acting elements which allow induction of the gene under abiotic stress. Some of the more important cis-acting elements found in *rd29a* are ABA-responsive elements (ABRE) which are transcriptional activators responsive to ABA treatments(Nakashima et al., 2006; Narusaka et al., 2003), and Dehydration- responsive elements (DRE), which are responsive to drought and osmotic stress(Nakashima et al., 2006; Stockinger, Gilmour, & Thomaschow, 1997). It has previously been described that multiple ABRE confer ABA responsiveness in

promoters of ABA-inducible genes, however a single ABRE is insufficient to elicit an ABA response (Skriver, Olsen, Rogers, & Mundy, 1991). The *rd29a* promoter is shown to have only a single ABRE, but ABRE interdependence with the Drought Responsive Element (DRE) in *rd29a* has been shown to induce a weak ABA response (Narusaka et al., 2003). Further investigation using qPCR on these *rd29a* and *35S* mediated knockdown lines to evaluate *PP2C* levels may be interesting to compare RNAi expression between the two promoters. Notably, it would be interesting to look into *AHG1* and *AHG3* which are genes whose expression levels are higher in dry seeds (Nishimura et al., 2007). *AHG1* has been shown to interact with the positive seed dormancy-regulating gene *DOG1* (Née et al., 2017; Nishimura et al., 2018).

To determine if constitutive and stress inducible knockdown of PP2C would correlate with enhanced drought tolerance, we tested our mutants in a low moisture assay. While drought is an increasingly prevalent problem, areas of extreme drought remain only a small part of agricultural areas (FAO land&water, 2014). As such, investigation of our mutants under constant low moisture stress would be more relevant to real world application than simply looking into survivability without water.

We found that both drought-inducible and constitutive PP2C knockdown lines had no growth penalties in well-watered conditions (Figure 4 C and E). However, in low moisture conditions, both *rd29a* and *35S*-mediated PP2C knockdown lines showed enhanced drought tolerance (Figure 4 D and E). This result is promising, because while our mutant lines both show enhanced drought tolerance, they do not show the growth penalty associated with PP2C knockout lines. Furthermore, by utilizing an assay more in line with the kind of drought more readily found in agriculture, results indicate that in *A.thaliana* , *35S* and *rd29a*-mediated PP2C knockdown result in enhanced drought tolerance without any well-watered drawbacks.

Interestingly, contrary to ABA responsiveness in germination and root elongation we did not observe any noticeable differences between *rd29a* and *35S* driven knockdown mutants. There is an increased response of the *rd29a*-mediated PP2C knockdown line in drought conditions as compared to ABA conditions. The enhanced drought response of *rd29a* mediated falls within our assumptions that multiple DRE would elicit a stronger drought response than a single ABRE would give as an ABA response. Interestingly, we did not find a significant growth penalty in well-watered conditions for the ABA hypersensitive triple knockout mutant *hab1-1 abi1-2 pp2ca* (Figure 4C and E). However, the triple knockout mutant did show enhanced drought tolerance (Figure 4D and E).

Further investigation of other homozygous *rd29a*- and *35S*-mediated knockdown lines in a same assay would be required. Furthermore, using qPCR on these lines to determine *PP2C* expression levels and RNAi expression would be also be interesting, as we have no clear link between gene expression of *PP2C*s and our phenotypes. Furthermore, comparing induction levels of *rd29a* under drought stress and ABA treatments would give us better insight into the different actions of DRE and ABRE in activating our knockdown.

PP2C knockout lines *abi1-1* and *aba1-2* show lack of sensitivity to ABA, and increased stomatal density(Rodrigues et al., 2013; Tanaka, Nose, Jikumaru, & Kamiya, 2013). It would be interesting to analyze stomatal density and index of our *rd29a* and *35S*-mediated knockout lines to determine if there is any difference in stomatal development due to reduced expression of PP2Cs. Furthermore, PP2C knockout mutants have enhanced ABA-associated stomatal closure (Merlot, Gosti, Guerrier, Vavasseur, & Giraudat, 2001; Rubio et al., 2009). Investigating transpiration rates of our drought-inducible and constitutive PP2C knockdown lines using the petiole feeding method described in Ceciliato et al,.(2019) would give us better insight into the responsiveness of our

mutants to ABA. There is a clear link between osmotic stress in plants and activation of DRE (K. Yamaguchi-Shinozaki & Shinozaki, 1994; Kazuko Yamaguchi-Shinozaki & Shinozaki, 1993), and while *rd29a* yields a mild response to ABA, *rd29a* has been shown to have a strong response to osmotic stress. Using whole plant gas exchange similar to the protocol described in Ceciliato et al,. 2019 with plants grown in hydroponics then subjected to an ABA or polyethylene glycol (PEG) would give us better insight onto the differences in ABA and osmotic stress in *rd29a*-mediated PP2C knockdown.

Effects of rd29a and 35S-mediated Clade A Type 2C Protein Phosphatases knockdown in B.napus

While modelling the RNAi for *B.napus* PP2Cs was done through *A.thaliana* orthologues and incomplete sequence annotations for *B.napus* (Figure 1), differences between the species remain. Notably, in *B.napus* the sequence similarities between the RNAi and the targeted PP2Cs was found to be Bn*ABI1* (chrA01 88%, chrC01 87.8% identity) and Bn*ABI2* (chrA10 77.6% identity), meaning we may have lowered targeting and expression of our RNAi in *B.napus* than in *A.thaliana*. As such, we looked into the different growth stages of *B.napus* to compare the *rd29a*-mediated PP2C knockdown in both *B.napus* and *A.thaliana*.

We found that drought-inducible PP2C knockdown lines show variable responses to ABA, with two of our lines showing enhanced response to ABA (lines 9 and 21), and one showing wild-type like ABA response (Figure 6). Interestingly, we found that one of our lines shows increased seed dormancy in control conditions (line 9) (Figure 6). Seed dormancy is regulated by PP2Cs, with *AHG3* and *AHG1* being shown to heavily involved with *DOG1*, a positive regulator of seed dormancy (Née et al. 2017). The DRE elements of the promoter for *rd29a* have been also shown to be strongly activated under cold stress (Msanne, Lin, Stone, & Awada, 2011; Nakashima, Yamaguchi-Shinozaki, & Shinozaki, 2014). We speculate that this specific line (line 9) which

shows enhanced seed dormancy under control conditions may have been more affected by the vernalization process due to a stronger insertional event, leading to a stronger PP2C knockdown. By testing our seeds in qPCR for PP2C gene expression in both cold and ABA conditions, we may have a better insight into the responsiveness the *rd29a* promoter for in either condition.

To determine if stress-inducible knockdown of PP2C would correlate to enhanced drought tolerance, we tested our *B.napus* mutants in a drought assay. We were uncertain of the degree of RNAi expression in *B.napus* transgenic lines. As such we sought to create an assay that would be most representative in differences in drought tolerance between genotypes. Previously in our lab, Dianne Pater had developed a protocol for rapid dry down drought assay using fritted clay. The advantages of this protocol was that the soil used was very uniform in its dry down (Dianne Pater unpublished), and created a stress that was well balanced between all genotypes in the pot. However, these conditions are not as effective in analyzing real-world adaptability of our mutants, as the protocol demands the soil be fertilized every other day, and mortality rate at the seedling stage is very high (Dianne Pater unpublished), resulting in low sample sizes.

We found that through the fresh weight in our drought assay, that two of our lines showed a trend toward being more drought tolerant (line 10 and 17) (Figure 7B). However, one of these lines (line 17) also showed a trend towards having some growth penalties under non-drought conditions (Figure 7B). While the assay was effective in maintaining moisture levels at a stable level across post (Figure 7A), high mortality rate of seedlings and stunting of growth as compared to soil conditions, render this assay's results inconclusive. Interestingly, the *B.napus* plants grown on fritted clay exhibited odd growing patterns, with certain leaves exhibiting a shriveled curled leaf phenotype in both well-watered and drought conditions. Furthermore, the sand-like quality of

the substrate caused compaction post-watering, leading to decreased root growth as compared to soil. Replicating the low moisture assay done for our *A.thaliana* mutants would be more appropriate to look at drought-tolerant phenotypes in our *B.napus.* Evaluation of PP2C levels at the end of the drought assay would allow us to have better insight on gene expression of these negative regulators, and the effectiveness of our knockdown. Similarly as proposed for *A.thaliana* mutants, assessment of stomatal conductance through petiole feeding of ABA, and stomatal density would be important to give us insight on whether our plants are more ABA-responsive when fully grown, and if there are any stomatal development was affected by the knockdown.

The Fritted clay approach towards looking into drought tolerance of *B.napus* plants was shown to give a uniform stress to the *rd29a*-mediated PP2C knockdown lines, however, issues of both high seedling mortality rates and lack of real-world applications lead me to believe that the drought assay developed for *A.thaliana* plants is a better method. We were able to generate ABA responsive *B.napus rd29a*-mediated PP2C knockdown lines, however the expression levels of the knockdown between lines stiil needs to be assessed. Finally, testing the same ABA responsive lines in the drought assay we developed for *A.thaliana* plants would allow us to observe if the enhanced ABA response would lead to increased drought tolerance.

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Appendix

Assessing *ropgef2* ABA responsiveness

INTRODUCTION:

The ROP family in plants are a group of Rho-like GTPases (ROP) responsible with regulating polarity of plant growth through the auxin signaling pathways, responses to plant hormones, and development of plant epidermal cells (D. Lin, Ren, & Fu, 2015; W. Lin, Tang, Anderson, & Yang, 2018). Activity of ROPs cycle between the GTP "on" state and the GDP "off" state allowing regulation of diverse signaling cascades, such as the FERONIA-mediated root growth hair or cell polarization (H. ming Wu, Hazak, Cheung, & Yalovsky, 2011). ROPs are regulated by a diverse group of proteins, such as Rho GTPase activating proteins (RopGAPs) (G. Wu, Li, & Yang, 2000; Yalovsky, Bloch, Sorek, & Kost, 2008), but one of the main groups of activators is a group of proteins known as Rho Guanine exchange factors (GEF) (Berken, Thomas, & Wittinghofer, 2005). GEF act as an "on" switch for ROP by catalyzing the GDP to GTP exchange. Recent studies have shown that GEFs have been implicated in the ABA signaling pathway through ROPs, such as GEF1 and GEF4 with ROP11 triggering ABA related stomatal closure(Li & Liu, 2012).

Interestingly, GEF2 (GEF2) has been reported to be involved in the ABA signaling pathway, with the knockout mutant being reported to be ABA-hypersensitive in germination(Zhao et al., 2015). GEF2 interacts with ROP2, ROP6 and ROP10, which have also been implicated with ABA signaling(Zhao et al., 2015). ROP11 has been shown to interact with *ABI2*, which is a Clade A Type 2C (PP2C) heavily implicated as a negative regulator in the ABA signaling pathway (Yu et al., 2012). To study GEF2's role in the ABA signaling pathway, we tested the *gef2* knockout line in ABA conditions in both germination and root elongation assays, and found no enhanced ABA responsiveness. To further test the role of GEF2 ,we knocked out *GEF2* in the ABA hypersensitive *gef1-4-10-14* background (Li et al., 2016) using

CRISPR-Cas9, in order to investigate if the quintuple mutant yields any additional ABA responsiveness as compared to the hypersensitive quadruple mutant.

MATERIAL AND METHODS

Root Growth assay

Sterilized *A. thaliana* seeds were cold treated for 4 days then grown in ½ MS for 5 days, and seedlings with similar primary root length were transferred to ½ MS (with or without 20 μM ABA) for 5 days. Primary root length was assessed using ImageJ (Rueden et al., 2017). Statistically significant differences between roots lengths was assessed using one-way ANOVA followed by Tukey's multiple comparisons test.

Germination assay

For *A.thaliana gef2* knockout line germination assays were performed as per Zhao et al., (2015). Growth conditions were the same as Pater, Mullen, McKay, & Schroeder, (2017). Pictures were taken daily for 6 days and cotyledon emergence was scored using ImageJ (Rueden et al., 2017)

gRNA for CRISPR design and plant transformation

CRISPR-Cas9 guide RNA were designed using CHOP CHOP v3 (Labun et al., 2019), and were designed to target exons 2 and 7 for the RopGef2 gene (At1G01700) (Table 3) Guide RNAs were inserted through Gibson Assembly by Dr. Jiyoung Park into a CRISPR-Cas9 plasmid (donated by

Dr. Yunde Zhao, unpublished). Competent *E. coli* cells were transformed with the plasmid DNA through heat shock and allowed to grow under selection for Kanamycin resistance. The plasmid DNA was then extracted and sequenced. Electro competent *A. tumefaciens* was then transformed with the construct and floral dipping method was used to transform *gef1-4-10-14* plants. mCherry positive seeds indicating successful transformation were selected for through fluorescence microscopy (Gao, Chen, Dai, Zhang, & Zhao, 2016). Homozygous mutants will be selected for using a combination of E1+E3 and E1+E7.(Table 3).

Table 3: Primers used for CRISPR-Cas9 *gef2* knockout

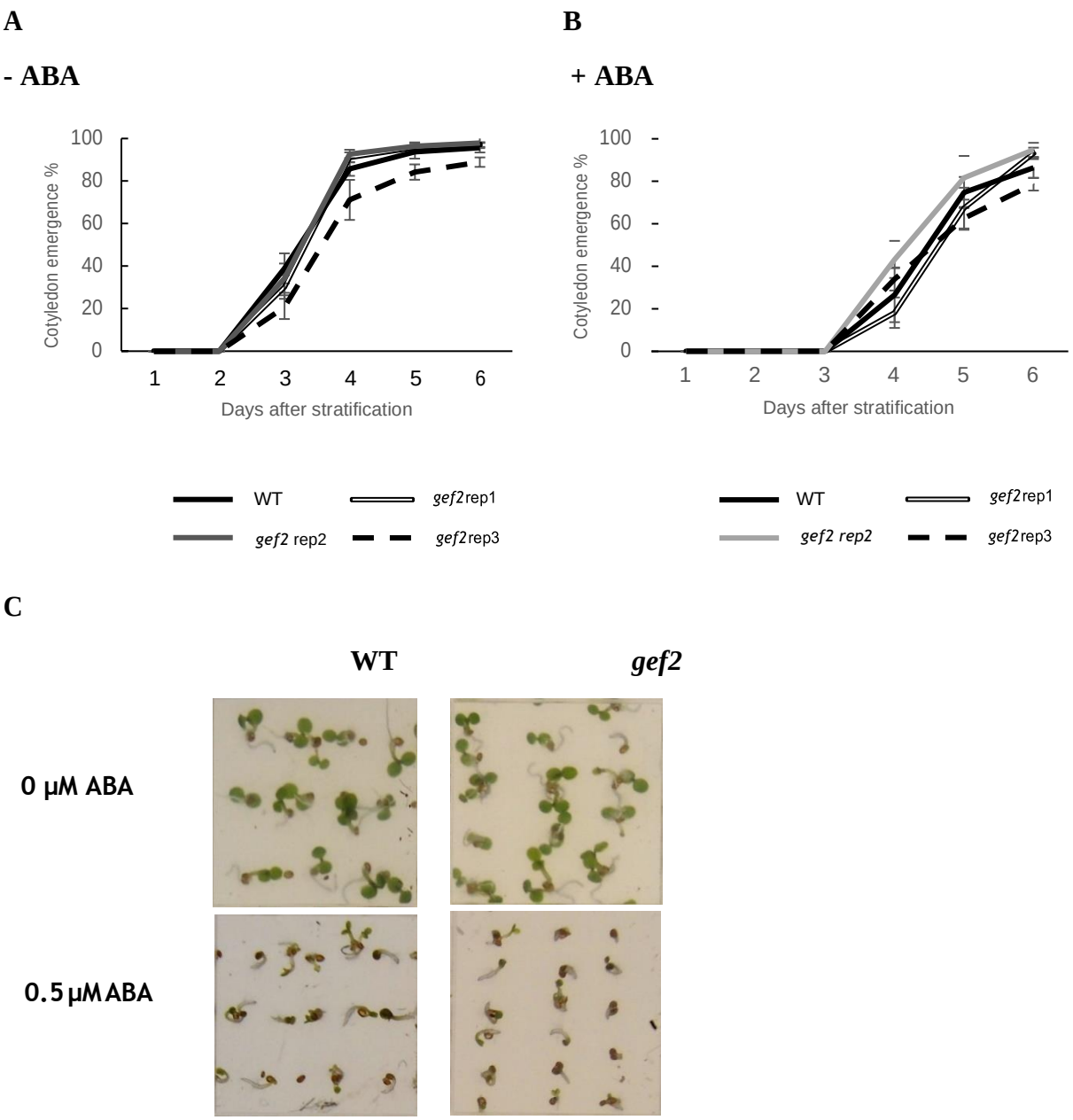
Primer Used	Gene	Purpose
CTAGAGTCGAAGTAGTGATTGTCATTCAGCA TCTGAAACTCGTTTTAGAGCTAGAAATAGC	AT1G01700	Guide RNA (Exon 2)
TGCTATTTCTAGCTCTAAAACAGAATTGTCT CAGACATCGTCAATCTCTTAGTCGACTCTA	AT1G01700	Guide RNA (Exon 7)
AAATCACGAAGAAAACGACGAT	AT1G01700	Exon 1 binding primer (E1)
TTTCAAGTCGAAGACAGTTGGA	AT1G01700	Exon 3 binding primer (E3)
AAGAACATCATCAATCCAAGCA	AT1G01700	Exon 7 binding primer (E7)

RESULTS:

The gef2 knockdown mutant is not hyper-sensitive to ABA in germination or root growth assay

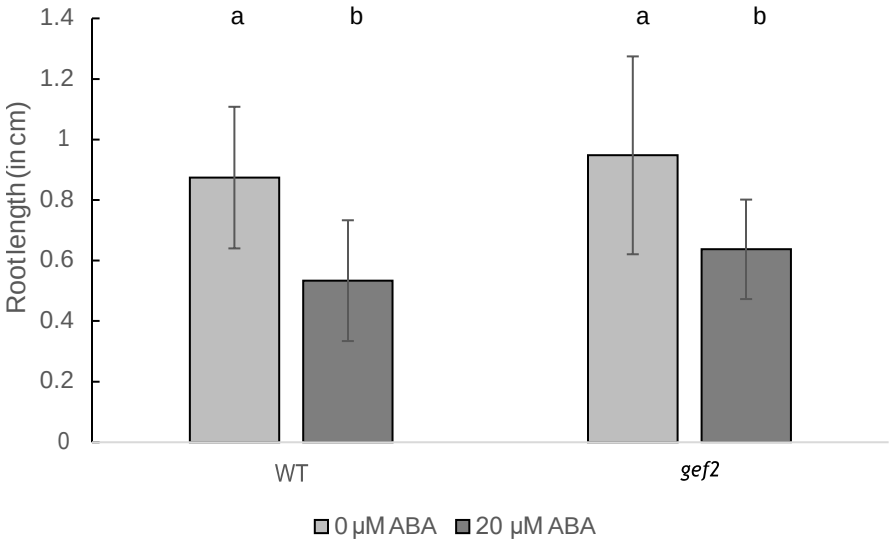
The Guanine Exchange Factor 2 (GEF2) has been reported to play a stabilizing role for Clade A Type 2C Protein Phosphatases, which are negative regulators in the drought response signaling pathway in plants (Zhao et al. 2015). The *gef2* knockout mutant (*SALK_130229*) has been reported to have enhanced ABA responsiveness in germination (Zhao et al. 2015). I found no significant difference in ABA responsiveness in this particular *gef2* knockout line during germination (Figure 7). We further investigated the ABA responsiveness of the *gef2* knockout mutant by performing a root elongation assay at the seedling stage. I transferred 4 day old seedlings to ½ MS plates containing 20 μM ABA for 5 days and then measured primary root growth. I found no significant difference between *gef2* mutants and wild type control plants (Figure 8).

Previously, the quadruple mutant *gef1-4-10-14* was shown to be ABA hypersensitive (Li et al., 2016). To determine whether *GEF2* may have some genetic redundancy with the *GEF1-4-10-14* we conceived to knockout *GEF2* in a *gef1-4-10-14* background (figure 9A). To do this, I generated CRISPR-Cas9 construct targeting *GEF2* exons 2 and 7 in a *gef1-4- 10-14* background, and selected for mCherry positive seeds using methodology proposed in Gao et al,. (2016) (Figure 9A and B). Once homozygosity is established, this mutant will be tested for ABA hypersensitivity in both germination and seedling stages to look for potential genetic redundancy of GEF2 with GEF1-4-10-14.



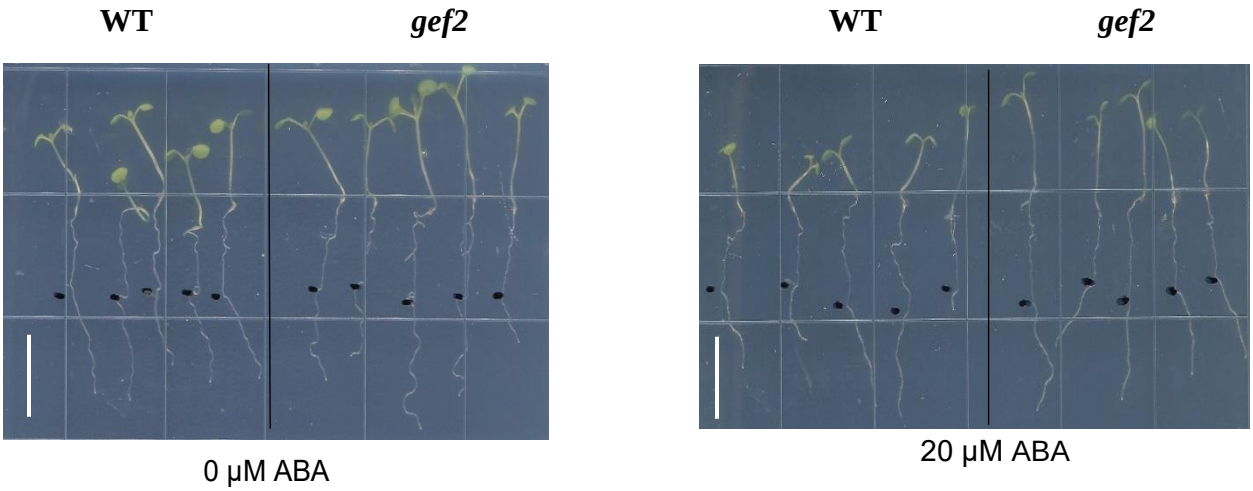
<u>Figure 8:</u> *gef2* shows no hypersensitivity to ABA as measured by cotyledon emergence. A Cotyledon emergence in ½ MS conditions for *gef2*. **B** Cotyledon emergence in 0.5 µM ABA for *gef2*. **C** Cotyledon emergence on Day 4 for WT and *gef2* in 0.5 µM ABA and ½ MS conditions. (n=3 biological replicates of 40-50 seeds, analyzed in each experiment per genotype)

A



B

C



<u>Figure 9:</u> ***gef2*** **shows no hypersensitivity to ABA as measured by root elongation. A** Root elongation assay on *gef2* mutants in 0 µM ABA and 20 µM ABA after 5 days of treatment. Error bars represent standard deviation. **B** Representative pictures of root lengths of WT and *gef2* in control conditions, seedlings were grown for 4 days in ½ MS and transferred to ½ MS for 5 days. (n= 3 biological replicates, each of 5 replicates of 4 seedlings) Bar represents 1 cm. **C** Representative pictures of root lengths of WT and *gef2* in ABA conditions, seedlings were grown for 4 days in ½ MS and transferred to 20 µM ABA plates for 5 days (n= 3 biological replicates, each of 5 replicates of 4 seedlings). Bar represents 1 cm. Letters represent differing significance as assessed by one-way ANOVA followed by a Tukey post-test

A

Guide RNA (Exon 2): TCATTCAGCA TCTGAAACTC

Guide RNA (Exon 7): ACGATGTCTGAGACAATTCT

B

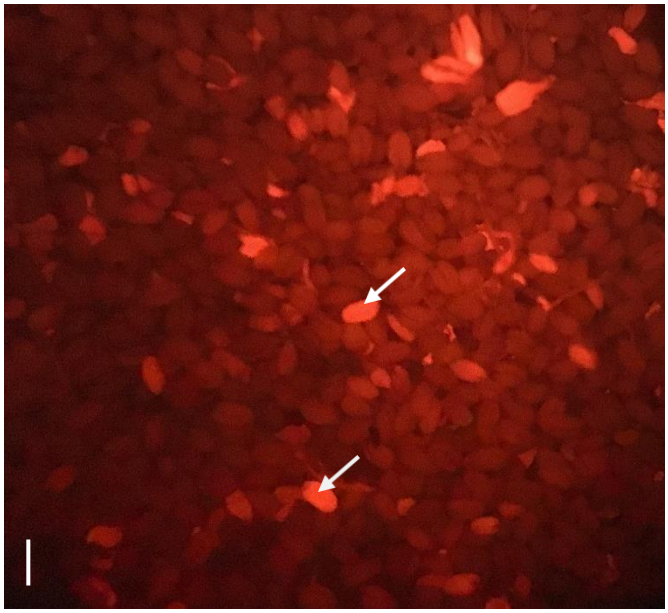


Figure 10: Generation of *gef2* CRISPR-Cas9 knockout in *gef1-4-10-14* background. **A** Targeting of Exon2 and 7 for Cas-9 cutting. Scale Bar represents 100bp. **B** Visual representation of mCherry positive seeds selected for Cas-9 activity. Arrows point out mCherry positive seeds. Scale bar represents 1 mm.

DISCUSSION:

Previous studies have shown the importance of ROP in the ABA signaling pathway, with ROP11 and ROP10 regulating the drought signaling pathway (Lee & Seo, 2019). ROP have been demonstrated to be tightly regulated by RopGEFs, with RopGEF1 and RopGEF4 in turn modulating the drought signaling pathway through their interactions with ROP11(Li & Liu, 2012). ROP11 has been reported to interact with the *ABI2*, playing a stabilizing role for the protein, and preventing interactions with the ABA receptor PYR/PYL 9. In this study we are looking into the ABA responsiveness of *GEF2*, where the knockout mutant has been demonstrated to have enhanced ABA responsiveness.

In order to test the ABA responsiveness of the *ropgef2* loss of function mutant, we isolated homozygous tDNA lines for the knockout. We found that there was no enhanced ABA responsiveness in the *gef2* knockout mutant in either germination or primary root growth (Figure 1 and 2). It was found that GEF1 and GEF4 directly interacts with PP2Cs and are localized in the guard cells, but the single knockout mutants *gef1* and *gef4* show no enhanced ABA responsiveness in germination. However, the double knockout mutants *gef1-4* showed enhanced ABA responsiveness in germination and enhanced arrest of root elongation following ABA treatment (Li & Liu, 2012). Genetic redundancy between GEF 1 and 4 allow compensation of each other in their roles in the ABA signaling pathway as single knockouts. The quadruple knockout mutant *gef1-4-10-14* shows higher ABA responsiveness, demonstrating additional genetic redundancy in GEFs regulatory function in the ABA signaling pathway. To investigate the role that RopGEF2 plays in the ABA signaling pathway, and the genetic redundancy of GEFs in regulating the ABA signaling pathway, we are generating a quintuple mutant *gef1-2-4-10-14* using CRISPR-Cas9 to knockout *GEF2* and then compare to the quadruple knockout mutant. We hypothesize that *ropgef2*

shows no enhanced ABA responsiveness in germination or root elongation due to the genetic redundancy of GEFs in the ABA signaling pathway. As such comparing GEFs that have already been shown to be involved in the ABA signaling pathway will help elucidate the role of *RopGEF2*.

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